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The role of post translational modification in regulating Human Papillomavirus (HPV)

E6 functions

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This thesis is submitted for the degree of Doctor of Philosophy in the
Faculty of Life Sciences of the Open University, UK



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ABSTRACT

The Human Papillomavirus (HPV) E6 oncoprotein from cancer-causing HPV types is highly multi-functional, capable of targeting many different cellular partners and pathways. Integral to this multi-functionality is its regulation by phosphorylation. Here I describe studies to firstly investigate when E6 is phosphorylated within the PDZ binding motif (PBM), and demonstrate a complex pattern of phosphorylation events which take place upon the exposure of the cells to different forms of stress. Most important of which is phosphorylation by kinases belonging to the core of the DNA Damage Response (DDR) machinery. Functionally this redirects E6 from interaction with PDZ substrates to association with 14-3-3 proteins. This in turn appears to contribute towards the ability of E6 to inhibit p53 transcriptional activity on a subset of p53 responsive promoters, thereby linking DDR signaling to the function of the E6 PBM. Functionally I have also been able to precisely dissect the sequence constraints within the E6 PBM governing phosphorylation by different kinases, PDZ recognition and interaction with different 14-3-3 isoforms. I also show that phospho-regulation of the E6AP ubiquitin ligase can also play a role in these pathways, and can be utilized and redirected by different HPV E6 oncoproteins with varying degrees of efficiency. Depending upon the specific HPV E6 protein, I also show the first evidence for phosphorylation at a site outside of the PBM and provide insights into the potential functional consequences thereof. Taken together, these studies shed new light on the role of E6 phosphorylation in a range of different biological activities, and begin to explain the multi-functional nature of the high risk HPV E6 proteins.

CHAPTER 1: INTRODUCTION

Cancer: A multifactorial disease

Cancer is a group of diseases that involve uncontrolled and deregulated cell growth with the potential to invade or spread to different parts of the body. The development of cancer involves various complex factors and steps. The hallmarks of cancer comprise six major biological activities. They include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Underlying these events, several other steps occur, such as genome instability, immune destruction and inflammation that further contribute towards the progression of malignancy (Hanahan and Weinberg 2011).

There are several factors that are responsible for cancer development. The majority of cancers are caused by genetic mutations resulting from causes in the natural environmental, such as exposure to ultraviolet (UV) light. Other factors, which could be defined as effects of the life-style environment, such as exposure to chemicals or gamma radiation also contribute to cause cancer. Importantly, approximately 12% of human cancers are caused by virus infection (Bouvard, Baan et al. 2009, de Martel, Ferlay et al. 2012, Humans 2012), in which viral oncogenic mechanisms and the host cofactors together contribute towards the development of malignancy (de Martel, Ferlay et al. 2012). The viruses that can contribute to cancer development include hepatitis B virus, (HBV) hepatitis C virus (HCV), Epstein Barr Virus (EBV), Human Papillomaviruses (HPVs), Human T lymphotropic Virus-1 (HTLV-1), HIV, Merkel Cell Polyomavirus (MCV) and Kaposi's sarcoma herpesvirus (KSHV). Viruses hijack the host cell machinery for their replication, immune evasion and survival. As a part of their life-cycle, human oncoviruses have evolved very powerful anti-apoptotic and proliferative strategies that can directly contribute to cancer hallmarks (Moore and Chang 2010).

Cervical cancer

Cervical cancer is the fourth most common cancer affecting women worldwide, after breast, colorectal, and lung cancers, and it is the most common cancer in the less developed countries of sub-Saharan Africa. Approximately 530 000 new cases of cervical cancer arise every year. It is also the fourth most common cause of cancer death (270 000 deaths in 2015) in women worldwide (WHO), and almost 90% of cervical cancer deaths occur in poorly developed regions. Cervical cancer ranks as the 2nd most frequent cancer among women in India. About 5% of women are estimated to have cervical HPV-16 and/or 18 infections at any given time, and 83.2% of invasive cervical cancers are attributed to HPV 16 or 18 infections (http://www.hpvcentre.net/statistics/reports/IND_FS.pdf).

Human papillomavirus and cancer

HPV is one of the most important causes of sexually transmitted disease worldwide. The virus is associated with cervical and other anogenital cancers, and with head-and-neck cancers (Garbuglia 2014, Wakeham and Kavanagh 2014). The high risk virus types are estimated to cause about 5% of the cancer burden worldwide, which includes 99% of cervical cancers, 25%–60% of head-and-neck cancers, 70% of vaginal squamous cell carcinomas, 88% of anal cancers, 43% vulvar and 50% of penile cancers (Gillison, Castellsague et al. 2014, Giuliano, Nyitray et al. 2015). Therefore, HPV continues to be an important subject for research, since the rate of HPV-related diseases is increasing day by day. There are over 200 HPV types, but only the so-called high-risk types are cancer-causing. HPVs are classified into 5 genera: α , β , γ , ν and μ , based on differences in their life cycles, their DNA sequences and also their disease association (Doorbar 2006, Bernard, Burk et al. 2010, Ekstrom, Bzhalava et al. 2011, Doorbar, Quint et al. 2012). It is now believed that viruses from the beta and gamma genera complete their life-cycle, causing asymptomatic infections in immunocompetent individuals, without causing any apparent disease phenotype (Forslund 2007, Nindl, Gottschling et al. 2007, Gottschling, Goker et al. 2009, Bottalico, Chen et al. 2011, Ekstrom, Bzhalava et al.

2011). HPVs from the alpha genus are categorized as either cutaneous types or mucosal types (Bernard, Burk et al. 2010), based on their ability to infect basal epithelial cells of the skin or inner lining of tissues. (Harwood, Spink et al. 1999, Burd 2003, Gillison and Shah 2003). The alpha type HPVs can also be grouped into high-risk and low-risk HPV types, based on their association with cervical cancer and its precursor lesions. Low-risk HPV types include types 6, 11, 42, 43, and 44. High-risk HPV types include 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (zur Hausen and de Villiers 1994, Bouvard, Baan et al. 2009, Bernard, Burk et al. 2010, de Villiers 2013). Squamous cell carcinomas (SCC) are the most common cervical cancers followed by adenocarcinomas (ADC) (Smith, Lindsay et al. 2007). HPV-16 is more commonly associated with SCC (55%) than with ADC (33%), while HPV-18 is more commonly associated with ADC (37%) than with SCC (13%) (Clifford, Smith et al. 2003, Smith, Lindsay et al. 2007). Infection with low-risk HPV types, such as HPV-6, -11, 42, -43 and -44, do not typically cause neoplasia but are responsible for the development of benign genital warts (Laimins 1993, Middleton, Peh et al. 2003). Some of the beta-type HPVs are also associated with cutaneous cancers. The patients presenting epidermodysplasia verruciformis (EV) have an increased susceptibility for developing such cancers, and a large number of different HPV types are found in the skin of those patients including HPV types 5, 8, 9, 12, 14, 15, 17, 19–25, 36–38, 46, 47, 49 and 50 (Cobb 1990) (Gewirtzman, Bartlett et al. 2008), although only HPV-5 and -8 are defined by the WHO as cancer causing in those individuals.

Among the alpha HR HPV types, HPV-16 and -18 are described as the most potent cancer-causing agents commonly found worldwide (Munger and Howley 2002, Munger, Baldwin et al. 2004, Snijders, Steenbergen et al. 2006), and these are especially responsible for invasive cervical cancers (Smith, Lindsay et al. 2007, Li, Franceschi et al. 2011).

Deregulation of viral life cycle: progression towards cervical Cancer

The virus particle infects basal keratinocytes, to which it gains access through micro-traumas in the epithelium; it then enters the cell and releases its viral genomic DNA, into the nucleus where is retained as an episome. The virus alters the normal differentiation process in the lower epithelial tissues during early infection, which results in the formation of low-grade squamous intraepithelial lesions (LSIL), clinically manifested as cervical intraepithelial neoplasia grade 1 (CIN1). The viral infection in this low-grade lesion may progress normally, or sometimes the virus becomes latent for several weeks to months. Generally, the virus is eliminated by the immune system from these differentiated cells. Detection of CIN1-like features can occur about 4 months after viral infection (Winer, Kiviat et al. 2005). Although 99% of cervical cancers are associated with High Risk HPVs (zur Hausen 1991, zur Hausen 1996), in the vast majority of cases the infection does not develop into the cancer. Most of the time, HPV infections are cleared by the host immune system. In certain cases, if the host immune system fails to clear the infection, or if the host is immunosuppressed, then the cells can remain persistently infected for many years. Such infected tissues can progress to severe dysplasia or high-grade squamous intraepithelial lesions (HSIL) also called CIN2 and CIN3, which can arise anywhere upto 14 months post-infection. Such severe dysplastic lesions may remain as HSIL, or advance to invade below the basement membrane, leading to local invasion (McMurray, Nguyen et al. 2001). The viral oncoproteins E6 and E7 play key roles in promoting the progression to cancer. In addition, accumulation of DNA damage and acquisition of chromosome instability contribute towards the development of cervical malignancy (Pett, Alazawi et al. 2004). The viral genome is often found integrated into the host genome in high-grade lesions, resulting in upregulation of the E6 and E7 viral oncoproteins, and loss of most of the other viral genes that are required for the viral life cycle (Schwarz, Freese et al. 1985, Shirasawa, Tomita et al. 1989, Jeon and Lambert 1995). It has been shown that approximately 70% of metastatic cells have integrated HPV genomes, which indicates that viral DNA integration is a common step in the pathogenesis of cervical malignancy (McMurray,

Nguyen et al. 2001). Although integration is more likely in HPV-18 positive tumours compared to HPV-16 positive (Cullen, Reid et al. 1991, Badaracco, Venuti et al. 2002, Fehrmann and Laimins 2003, Vinokurova, Wentzensen et al. 2008). The preferred sites for viral genome integration into the host chromosome are called common fragile sites (CFS); the suggested CFS include FRA13C (13q22), FRA3B (3p14.2), and FRA17B (17q23) (Thorland, Myers et al. 2003). The E2 ORF is identified as the preferential site of integration, since it is more commonly disrupted or deleted than any other site (Badaracco, Venuti et al. 2002). It is possible that integration can happen at any point in the HPV genome, but only those that disrupt E2 have any growth advantage for the cell. The E2 protein negatively regulates expression of E6 and E7 oncoproteins only when the genome is integrated (Bechtold, Beard et al. 2003), therefore, loss of E2 during integration results in increased expression of the transforming E6 and E7 oncoproteins (Romanczuk and Howley 1992). Thus, integration of the HPV genome results in the enhanced and deregulated expression of the E6 and E7 viral oncogenes, which further promotes cellular transformation.

Little is known about the process whereby HPV genomes are integrated into the host genome. However, several studies have suggested that the DNA damage response may play a role in HPV integration. A study in W12 cells, which stably express HPV16 episomes, demonstrated that when double strand breaks (DSBs) were generated by depletion of Ku70 (a crucial mediator of Non Homologues End Joining), new HPV16 viral integration events occurred (Winder, Pett et al. 2007). It has also been shown that the activity of DNA-dependent protein kinase (DNA-PK), an important molecule involved in DSB repair, was found to be significantly reduced in patients with cervical cancer (Someya, Sakata et al. 2006). These studies indicate that DSBs may be associated with HPV episome loss and integration in cervical cancer. In the case of Hepatitis B Virus, it has been shown that integration frequency increases with DNA damage, and that viral DNA integration coincides with severe dysplasia, leading to hepatocellular cancer (Dandri, Burda et al. 2002). Therefore, it is reasonable to expect that DNA damage to both the viral episome and the host

genome might enhance the integration of viral DNA into that of the host. Another potential mechanism by which HPV integration could occur is ‘Inflammation-mediated DNA damage’. Indeed, inflammation has been implicated in the progression of a variety of cancers. It has been suggested that excessive amounts of reactive oxygen species (ROS) are generated during chronic inflammation and these play a critical role in promoting DNA damage, ultimately leading to carcinogenesis (Kawanishi, Hiraku et al. 2006). In the case of HPV-associated cancers, inflammation would also facilitate the integration of the viral genome by inducing breaks in both the viral and host genomes. It is likely that there is epigenetic modulation, such as DNA methylation and chromatin modifications, of the integration site that further determines whether the integration site is active or silenced (Chaiwongkot, Vinokurova et al. 2013, Groves, Knight et al. 2016).

Additionally, the initial integration event is thought to occur in a cell that also harbors extrachromosomal HPV genomes expressing E1 and E2. The E1 and E2 proteins may cause overamplification, or onion skin replication, of the integrated HPV genome. This results in generation of heterogeneous replication intermediates that serve as substrates for recombination and repair, resulting in rearrangements, deletions, and amplification of viral and host sequences (Kadaja, Sumerina et al. 2007, Kadaja, Isok-Paas et al. 2009). This can lead to genomic instability of the host region at the common fragile sites, and further continued expression of E6/E7 contributes to genetic instability. Therefore, many events are likely contributed to the HPV integration event and its subsequent evolution, which is a strong driver of carcinogenesis. (Figure 1)

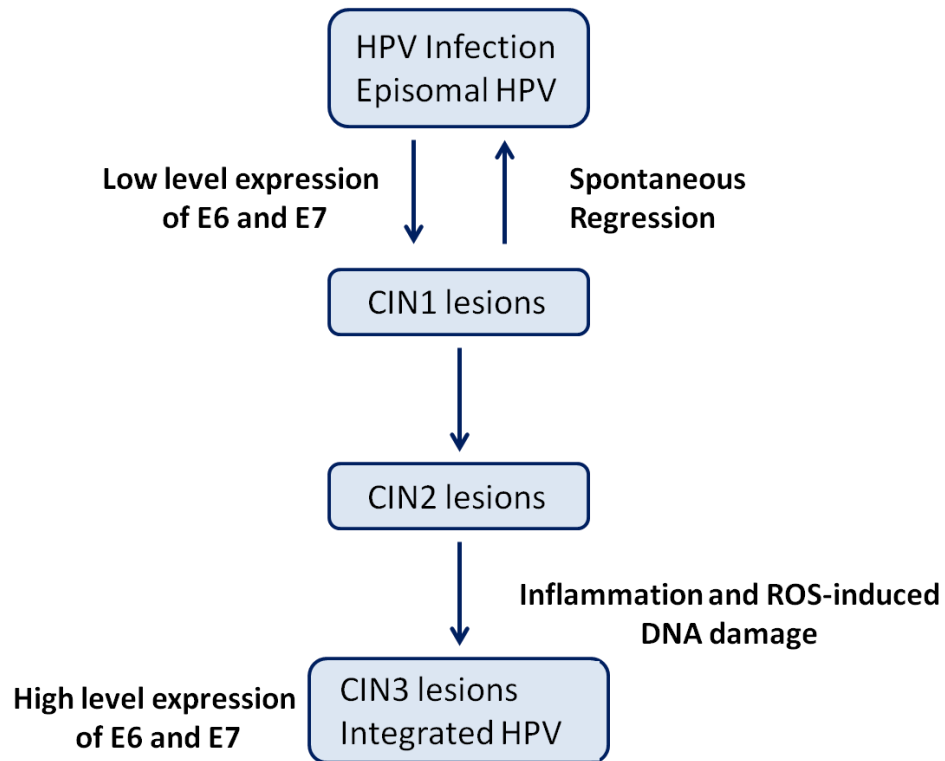


Figure 1. Schematic representation of the progression of cervical malignancy.

HPV infection of cells in the lower levels of the cervical epithelium leads to an alteration of the normal differentiation process in the lower epithelial tissues, which results in the formation of low-grade squamous intraepithelial lesions (LSIL), clinically manifested as cervical intraepithelial neoplasia grade 1 (CIN1). This usually has a high rate of regression back to normal cells. CIN2 represents a mixture of low- and high-grade lesions and, thus, further progress towards high-grade dysplasia, CIN3. At this stage, excessive amounts of reactive oxygen species (ROS) are generated during chronic inflammation and which may play a critical role in promoting DNA damage, ultimately leading to carcinogenesis. At this stage, the HPV genome is often found integrated into the host genome.

HPV genome: Structure, organization and function

HPVs are small, non-enveloped viruses, 55 nm in diameter. They have an icosahedral capsid composed of 72 capsomers, with two capsid proteins, L1 and L2. Each capsomer is composed of 360 copies of pentamer of the major L1 capsid protein. Each virion capsid also contains upto 72 copies of the minor capsid protein, L2 (Sapp, Volpers et al. 1995, Buck, Cheng et al. 2008). The HPV genome consists of a double-stranded circular DNA molecule of approximately 7,900 bp, associated with histones which forms chromatin-like structures (Favre 1975). As shown in Figure 2, HPV has approximately eight open reading frames (ORFs), and all protein-coding sequences are transcribed from one single strand. The genome is functionally divided into three regions, the Upstream Regulatory Region (URR), the Early region and the Late region. HPV DNA replication is regulated by controlling the transcription of the ORFs, and this regulation is mediated by the p97 promoter in HPV-16, the p99 in HPV-31 and the p105 in HPV-18, along with many enhancer and silencer sequences. The URR also contains an origin of DNA replication, around which are the binding sites for E1 and E2 proteins. The Early region consists of ORFs E1, E2, E4, E5, E6, and E7, often referred to as early genes, which are involved in viral replication. The Late region contains the ORFs encoding the structural capsid proteins, L1 and L2, and are expressed during the later phases of the viral life cycle. Extensive alternative splicing also increases the coding capacity of the genome.

The E1 protein is about 600 to 650 amino acids long depending on the papilloma virus (PV) type, and is the only PV encoded enzyme that possesses DNA helicase activity. E1 serves various functions that are required for the initiation of viral DNA replication, including ori-specific DNA-binding activity, DNA-dependent ATPase activity and helicase activity (Ustav, Ustav et al. 1991, Wilson and Ludes-Meyers

1991, Seo, Muller et al. 1993, Thorner, Lim et al. 1993, Lusky, Hurwitz et al. 1994). The E2 transcriptional activator protein is much smaller than E1, being 350 to 500 amino acids in length depending on the PV type. The interaction between the E1 and E2 proteins results in the formation of a complex called the 'E1-E2-ori complex' on the origin of replication (ori) (Sedman and Stenlund 1995, Sedman and Stenlund 1996). This interaction has several consequences, one of which is that the specificity of binding of the E1 protein to the ori is enhanced significantly. Moreover, in the presence of E2, the DNA-binding capacity of E1 is substantially higher (Sedman and Stenlund 1996).

Transcriptional regulation of early viral gene products is a central regulatory event for viral infection. The initiation of transcription is activated by host cell transcription factors, which bind to the URR upstream of the early promoters of high-risk HPVs. The activity of these promoters is further modulated by the viral E2 protein through its interaction with four highly conserved E2-binding sites (E2-BS) located in the URR of all high-risk HPVs, which are essential for a productive viral life cycle. Binding of E2 to promoter BS1 affects recognition of the neighboring TATA box by TBP, whilst binding of E2 to BS2 and BS3 may contribute to promoter repression by competition with cellular transcription factors such as SP1 (Demeret, Yaniv et al. 1994, Demeret, Desaintes et al. 1997). E2 binding at BS4 leads to an upregulation of viral early gene expression and E2 binding at BS3 is essential for viral DNA replication. Thus, at low concentrations of E2, BS4 is occupied, therefore, the E6/E7 promoter is activated (Romanczuk, Thierry et al. 1990). Similarly, as E2 expression increases, the E6 promoter activity is suppressed (Steger and Corbach 1997), concomitant with E2 occupation of BS1 and BS2 (Sanders and Maitland 1994). Therefore, the E2-DNA-binding events correlate with an increased E2 expression and co-ordinate early gene expression (Thierry and Howley 1991, Bouvard, Storey et al. 1994). E2 transcriptional activity is controlled by p300-mediated acetylation of lysine 111. This represents a novel mechanism by

which papillomavirus gene expression is regulated. E2 also plays a pivotal role in ensuring the correct distribution of viral episomes to the daughter cells during mitosis and cell division and this is achieved by tethering the viral episomes to the host chromosome through its association with the cellular Brd4 protein (McKinney, Kim et al. 2016).

The HPV E4 protein is approximately 17kDa protein, and is synthesised as an E1^{E4} fusion protein, as a result of mRNA splicing (Chow, Reilly et al. 1987, Ozbun and Meyers 1997, Milligan, Veerapraditsin et al. 2007, Wang, Meyers et al. 2011); although designated as an early protein, its expression is mostly detected later in infection, after the activation of the late promoter (Doorbar 2013). Although there is considerable debate on the role of E1^{E4} during the viral life cycle, studies on high-risk HPV types-16, -18 and -31 have suggested multiple roles during the late stages of the viral replication cycle. The leucine structure within E1^{E4} protein has been shown to be important for E4 protein self-association, and this confers on E4 the ability to form structures resembling amyloid fibres, allowing manipulation of the host cell cytokeratin network potentially for subsequent virion release (McIntosh, Martin et al. 2008, McIntosh, Laskey et al. 2010). Furthermore, epithelial cells expressing E4 from HPV-16, HPV-18 and HPV-31 were shown to undergo cell cycle arrest in G2 phase, owing to the inhibition of Cyclin/Cdk1 nuclear accumulation (Davy, Jackson et al. 2002, Davy, Jackson et al. 2005, Knight, Turnell et al. 2006). More recent studies showed that loss of E4 has marked effect on viral genome amplification and L1 expression upon differentiation. However, HPV-16 showed a much clearer E4 dependency for viral genome amplification than HPV-18 (Egawa, Wang et al. 2017), the reasons for which remains to be determined.

The E5 protein is 75 to 100 amino acids long depending on PV type, and is a transmembrane-associated hydrophobic protein, which binds with intracellular

membranes, most notably those of the endoplasmic reticulum, Golgi apparatus, and perinuclear region. The E5 protein is frequently referred to as the HPV minor oncoprotein. HPV16 E5 has been shown to induce anchorage-independent growth in human keratinocytes and in murine fibroblasts (Pim, Collins et al. 1992). High levels of E5 expression in the transgenic mouse model has been shown to induce epithelial hyperproliferation, resulting in tumor formation in the skin. Furthermore, these mice showed increased dysplastic disease in the cervix (Genther Williams, Disbrow et al. 2005, Maufort, Williams et al. 2007, Maufort, Shai et al. 2010). E5 has also been involved in activation of epidermal growth factor receptor (EGFR) signaling, thereby enhancing extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) activity (Straight, Hinkle et al. 1993). In addition, E5 perturbs maturation of the endosome (Disbrow, Hanover et al. 2005), resulting in delayed EGFR degradation (Supryniewicz, Krawczyk et al. 2010), and E5 expression has also been associated with reduced levels of apoptosis (Oh, Kim et al. 2010) and immune evasion (Ashrafi, Haghshenas et al. 2005). Recently, E5 has been classified as viroporin-a channel-forming viral membrane protein. Thus the function of E5 is appears very closely linked to various vesicle trafficking pathways, and its loss from the viral genomes has a clearly deleterious effect on the viral life cycle (Wetherill, Holmes et al. 2012, Royle, Dobson et al. 2015).

E6 is a small protein of around 150 amino acids, containing four zinc-binding Cys-X-X-Cys (CXXC) motifs, whereas E7 is a small zinc-binding protein of 100 amino acids containing two CXXC motifs. Both E6 and E7 play the role of major oncoproteins, and will be discussed in more depth later in this chapter.

The viral capsid proteins L1 and L2 are approximately 500 amino acids long and are expressed during late stages of infection.

All of these genes are coordinately expressed during the viral life cycle during the differentiation of the infected epithelium. The roles of these genes during different phases of the virus life cycle are described below and are illustrated in Figure 3.

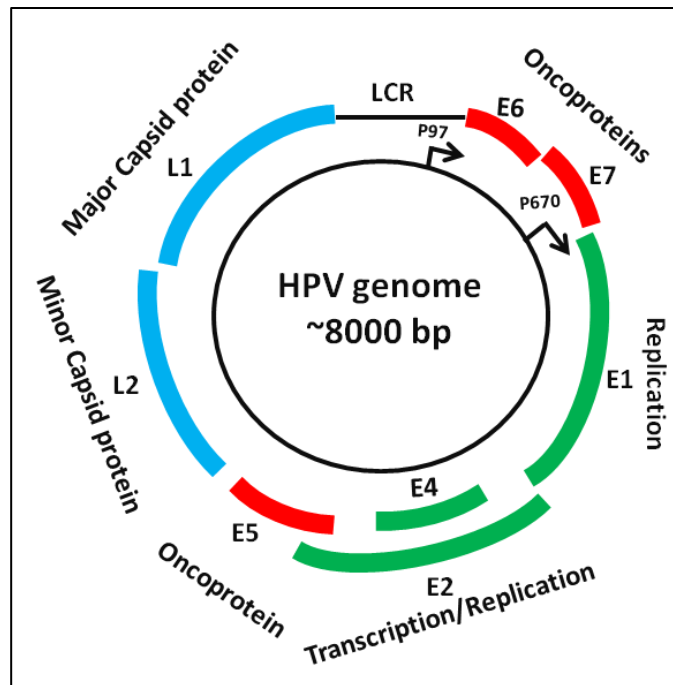


Figure 2. Typical HPV genome organization, represented by the HPV-16 genome, showing the different ORF of the virus genes.

The HPV genome contains a non-coding upstream regulatory region (URR), also called the long control region (LCR), of about 400 to 1,000 bp long. It harbors an origin of replication (ori), a promoter for early genes, four E2 binding sites and at least 20 other binding sites for host cell transcription factors, many of which are specific for epithelial cells. The Early region, encodes the viral E1, E2, E4, E5, E6 and E7 proteins. E1 acts as a helicase and binds to the ori for initiation of viral DNA replication. E2 functions as a viral transcription factor, and also regulates the expression of the E6 and E7 proteins. E5 is the minor oncoprotein and interacts with the EGFR and also suppresses host MHC I/II gene expression. E6 and E7 are the major oncoproteins and are essential throughout the viral life cycle and for the development of malignancy. The Late region encodes the structural capsid proteins: L1 and L2. E4 is also a late protein, and interacts with the host cytoskeleton, favoring the release of new viral particles.

Different stages of productive viral life cycle

Viral access to epithelium

HPVs are exclusively intraepithelial pathogens and their productive viral life cycle is intimately dependent upon the keratinocyte differentiation program. Infection by papillomaviruses requires the virus to access the dividing epithelial basal layer, where infection is speculated to occur through micro abrasions in the epithelium (Doorbar 2005). It is believed that virus infects basal keratinocytes, which possess a stem cell phenotype. The means of virus entry into the basal cells appears to be diverse in different HPV types, for example, HPV-31 entry is mediated by a caveolin-dependent mechanism (Smith, Campos et al. 2007), while HPV-16 entry is mediated by a clathrin- and caveolin-independent pathway (Spoden, Freitag et al. 2008, Spoden, Kuhling et al. 2013).

Studies in HR HPVs have shown that the epithelial cells around the squamo-columnar junction of the cervical transformation zone are more susceptible to HPV infection and cancer progression (Grayson, Rhemtula et al. 2002, Herfs, Yamamoto et al. 2012). The viral capsid proteins play a major role during infectious entry. The virus requires the presence of certain cellular receptors, such as heparin sulphate proteoglycans (HSPGs) and α -integrins (Joyce, Tung et al. 1999, Giroglou, Florin et al. 2001, Shafti-Keramat, Handisurya et al. 2003, Dasgupta, Bienkowska-Haba et al. 2011). Following the interaction of the virions with the host cell receptors, the capsid undergoes a conformational change, which leads to exposure of the L2 amino terminus, leading to its cleavage by secreted furin (Richards, Lowy et al. 2006). The viral particles are then internalised via endocytosis after the attachment (Selinka, Giroglou et al. 2002, Bousarghin, Touze et al. 2003, Day, Lowy et al. 2003, Culp and Christensen 2004). The internalization step also appears to require the active proliferation of the target cells (Broniarczyk, Ring et al. 2018). After entering the cell, the viral particles are transported and processed via the endocytic transport

machinery. In the initial steps, the viral capsid begins to disassemble as a result of acidification of endocytic compartments (Smith, Campos et al. 2008), this mediates the cytosolic exposure of the L2 proteins (DiGiuseppe, Keiffer et al. 2015) and allows L2 to associate with various cellular proteins that are part of endocytic sorting pathways, including the sorting nexin family of proteins, components of the retromer, and also the ESCRT complex (Bergant Marusic, Ozbun et al. 2012, Broniarczyk, Bergant et al. 2014, Pim, Broniarczyk et al. 2015, Popa, Zhang et al. 2015). Interaction of L2 with SNX17, which is involved in endosomal recycling pathways, helps L2 in escaping lysosomal degradation (Kamper, Day et al. 2006, Day, Lowy et al. 2008, Smith, Campos et al. 2008, Dabydeen and Meneses 2009). In the later steps of endocytic maturation, the L2-DNA complex is separated from L1, which is achieved via cyclophilins (Bienkowska-Haba, Williams et al. 2012). Most of the L1 protein is degraded in the lysosome (Spoden, Freitag et al. 2008, Buck, Day et al. 2013), but a small percentage of it remains associated with the L2/vDNA. At the same time, L2 mediates the transport of viral DNA through the trans-Golgi network to the host nucleus. Nuclear entry is strictly dependent on mitosis (Sun, Frazer et al. 1995, Florin, Becker et al. 2004, Bossis, Roden et al. 2005, Pyeon, Pearce et al. 2009, Broniarczyk, Massimi et al. 2015). Following nuclear envelope breakdown during mitosis (Aydin, Weber et al. 2014), the L2-DNA complex, plus the small amount of residual L1, is localised to PML bodies, where initiation of viral transcription is believed to occur (Day, Baker et al. 2004, DiGiuseppe, Bienkowska-Haba et al. 2017).

Viral establishment and maintenance

After infection of basal cells, a round of viral DNA replication takes place that appears to be independent of the cell cycle, and which amplifies the viral copy-number to around 50 to 100 copies per cell. After this round of DNA replication, the viral genome is established as a stable episome within the cell, and this is mediated by expression of the viral replication proteins, E1 and E2 (Wilson, West et al. 2002). The E1 protein is the viral origin-binding protein, recognizing and binding the viral

origin of replication (ori), a palindromic motif in the non-coding region of the viral genome (Dell, Wilkinson et al. 2003). E2 binding near the ori recruits the E1 protein, to the viral origin; this leads to the binding of other cellular proteins necessary for DNA replication, including DNA polymerase α -primase and replication protein-A (Loo and Melendy 2004). Papillomavirus genome replication is totally dependent upon these components of the cellular DNA synthesis machinery. The viral genome replication in the basal cells occurs together with the cellular DNA during S-phase synchrony. E2 is not only involved in viral DNA replication, but is also involved in transcriptional regulation. It can regulate the viral Early promoter, and controls expression of the viral oncogenes E6 and E7. It is not yet clear whether E2-mediated transcriptional regulation is important in basal epithelial cells, as the nucleosomal structure of the viral DNA may not be compatible with efficient activation of the Early promoter (Bechtold, Beard et al. 2003). Knockout mutants in the viral genome have shown that both E1 and E2 are required for viral genome maintenance in the basal layer (Stubenrauch, Lim et al. 1998). The increased proliferation of suprabasal epithelial cells is linked to the expression of the viral oncogenes, E6 and E7, in cervical lesions caused by HPVs. During the natural course of infection, the activity of E6 and E7 causes an expansion of replication competent cells, in order to enhance the production of progeny virions. For the replication of viral episomes in the suprabasal cells, E6 and E7 play an important role by driving the cells into S phase. It is important to note that these events occur in cells that are terminally differentiating and would have normally exited the cell cycle (Sherman, Jackman et al. 1997). Differentiation is delayed in these cells and cells are maintained in a pseudo-S-type state followed by G2/M phase where viral genome amplification occurs. The mechanism by which HPVs disrupt the cell cycle control is well-understood. E7 associates with pRb (retinoblastoma protein) and other pocket protein family members (p130, pRB and p107), leading to the disruption of the association between pRb and the E2F transcription factor (Dyson 1998). Additionally, E7 abolishes the activities of histone deacetylases (Brehm, Nielsen et al. 1999) and the cyclin-dependent kinase inhibitors p21 and p27 (Zerfass-Thome, Zwerschke et al.

1996, Funk, Waga et al. 1997, Jones, Alani et al. 1997), which helps to promote cell cycle progression and transcription. In high-risk HPV type infections, a key role of E6 is its association with p53, which mediates p53 ubiquitination and degradation (Scheffner, Werness et al. 1990, Scheffner, Munger et al. 1991). The reduction of p53 levels in the basal layer contributes to perturbation of p53 transcriptional activity, which subsequently reduces levels of the Notch receptor on the cell surface. Since Notch signaling plays a critical role in regulating basal cell density and commitment to differentiation, HPV-induced reduction in Notch activity thereby provides the cells with a competitive advantage over its neighboring uninfected cells, thus favoring expansion of infected basal cells (Kranjec and Doorbar 2016). Degradation of p53 also protects cells from growth arrest or apoptosis in response to the E7-mediated cell-cycle entry in the mid-epithelial layers.

Viral packaging and release

Packaging of virus genome into infectious particles is the final stage of the productive viral life cycle. After the onset of genome amplification, the capsid proteins L1 and L2 start to accumulate in the cell (Florin, Sapp et al. 2002) and the assembly of infectious virions in the upper epithelial layers also requires the activity of E2 (Day, Roden et al. 1998). It is thought that recruitment of the L1 protein to PML bodies by L2 enhances capsid protein packaging ability (Zhou, Stenzel et al. 1993, Stauffer, Raj et al. 1998, Florin, Sapp et al. 2002), and some HPV L2 proteins can associate directly with the nascent viral DNA (Fay, Yutzy et al. 2004). The virus, after its escape from the epithelial cells, must survive in the harsh extracellular environment, and this is thought to be mediated by the E1^{E4} protein. The amyloid fibres that are formed with the help of transglutaminase 3, further help to disrupt the normal cornified envelope of the upper epithelial cells, thus supporting the dispersal of new virions (Brown, Kitchin et al. 2006, McIntosh, Martin et al. 2008).

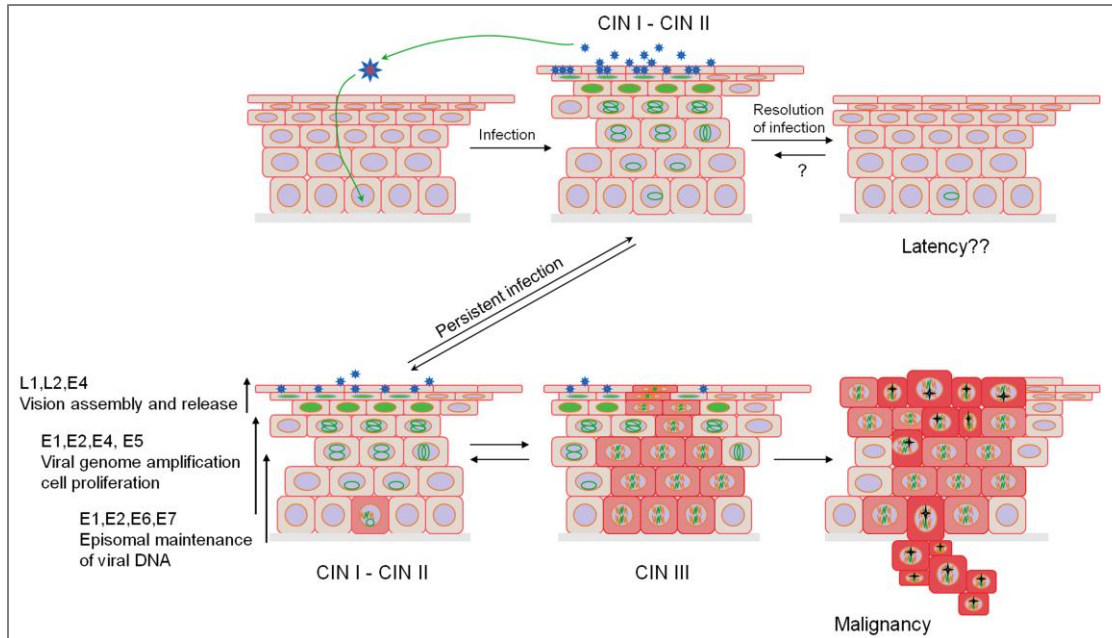


Figure 3. Productive life cycle of HPV and HPV-induced malignancy.

Human papillomaviruses infect the basal cells through micro-ruptures in the epithelium. Uninfected epithelium is shown on the left in green and HPV-infected epithelium is shown in the middle. The viral genomes stay in the nucleus as low copy-number episomes, and expression of the viral early genes begins. After the division of basal cells, some newly formed cells move up in the epithelium and start to terminally differentiate. In these differentiated HPV-positive cells the productive phase of the viral life cycle takes place. Activation of E6 and E7 allows viral genome amplification in cells that normally would have exited the cell cycle. In the later phase, the L1 and L2 capsid proteins are synthesized and the viral genome is encapsidated; ultimately mature virions are released from the epithelium. On the right side, the HPV-induced malignant transformation of epithelium, often as a consequence of a persistent infection is shown. At this stage, elevated levels of E6 and E7 oncoproteins are observed.

The HPV Oncoproteins

Cervical cancer does not develop in every individual who is infected with high-risk HPV types. When the immune system of an individual fails to clear the HPV infection, it can develop into a malignancy with the viral DNA frequently integrated into the host genome, resulting in the loss of E1, E2, E4 and E5 together with uncontrolled expression of the E6 and E7 oncoproteins (Moody and Laimins 2010, Doorbar, Egawa et al. 2015). So far many interacting partners of the E6 and E7 oncoproteins have been identified, which contribute in different ways to both the virus life cycle and malignant progression.

HPV E7

E7 is a small polypeptide of approximately 100 amino acids, exists as a dimer, localized in the cytoplasm (Smotkin and Wettstein 1987, Huh, Zhou et al. 2007, Nguyen, Eichwald et al. 2007, Ressler, Scheiden et al. 2007), and can also be found in the nucleus (Sato, Watanabe et al. 1989, Greenfield, Nickerson et al. 1991). E7 was the first HPV protein to be defined as an oncoprotein that could induce cell transformation (Kanda, Furuno et al. 1988, Phelps, Yee et al. 1988, Vousden, Doniger et al. 1988, Watanabe, Kanda et al. 1989). E7 contains three conserved regions (CRs); CR1 and CR2, found at the N-terminus, and the more C-terminally located CR3, as shown in Figure 4. The CR2 domain contains a conserved Leu-X-Cys-X-Glu (LXCXE) motif, through which it binds the Pocket protein family, and Casein Kinase II (CKII) phosphorylates the HPV-16 E7 at amino acid residues Serine-31 (S31) and S32, which reside in close proximity to the LXCXE motif (Firzlaff, Galloway et al. 1989, Barbosa, Edmonds et al. 1990). E7 is also phosphorylated at S71 by an unknown kinase, during the S phase of the cell cycle (Massimi and Banks 2000). The CR3 domain of E7 contains two CXXC motifs, which form a zinc-binding domain that is required for E7 dimerization (Barbosa, Lowy et al. 1989, McIntyre, Frattini et al. 1993, Clemens, Brent et al. 1995).

E7 is a multi-functional protein, able to interact with a very large number of cellular proteins, in many cases disturbing their normal functions. The E3 ubiquitin ligase complex, SCF (Skp-Cullin-F-box) is an interacting partner of E7 for its own ubiquitination and proteolysis (Oh, Kalinina et al. 2004), while the N-terminus of E7 associates with neddylated cullin 2 in order to induce the ubiquitination and degradation of the retinoblastoma tumour suppressor (pRB), (Huh, Zhou et al. 2007). In addition to pRB, E7 binds to its related pocket proteins, p107 and p130 (Lee, Russo et al. 1998, Gonzalez, Stremlau et al. 2001, Helt and Galloway 2001) through the LXCXE motif. These proteins have been shown to be involved in regulating cellular proliferation, differentiation and apoptosis (Dyson, Howley et al. 1989, Munger, Werness et al. 1989, Dyson, Guida et al. 1992, Dyson 1998). High-risk HPV E7 proteins also contribute to cell cycle dysregulation through multiple other mechanisms by associating with cyclins, and cyclin-dependent kinase inhibitors (CKIs) and regulating their activity. Cyclins E and A, both of which are under the control of E2F, are found overexpressed in E7 expressing cells (Zerfass, Levy et al. 1995). HPV-16 E7 also interacts with and abrogates the growth-inhibitory activities of the CKIs p21^{Cip1} (Funk, Waga et al. 1997, Jones, Alani et al. 1997) and p27KIP1 (Zerfass-Thome, Zwierschke et al. 1996). The ability of the HPV E7 oncoprotein to overcome p21^{Cip1}-mediated inhibition of cdk2 activity during keratinocyte differentiation contributes to the ability of E7 to allow cellular DNA synthesis in differentiated keratinocytes. (Favre, Breitburd et al. 1977, Jones, Alani et al. 1997, Swindle and Engler 1998, Noya, Chien et al. 2001, He, Staples et al. 2003). Both p21 and p27 have also been implicated in TGF- β -mediated growth inhibition (Elbendary, Berchuck et al. 1994, Polyak, Kato et al. 1994, Datto, Li et al. 1995) and thus inactivation of CKIs by HPV E7 may contribute to TGF- β -mediated growth arrest.

Additionally, E7 also interacts with class I histone deacetylases (HDACs) (Brehm, Nielsen et al. 1999, Longworth and Laimins 2004). Studies in HPV-31 have reported that the interaction between E7 and HDACs results in increased levels of E2F2-mediated transcription and replication in differentiating cells (Longworth, Wilson et

al. 2005), potentially favoring S-phase progression. E7 can also associate with histone acetyl transferases (HATs), such as p300, pCAF, and SRC1 (Huang and McCance 2002, Avvakumov, Torchia et al. 2003, Bernat, Avvakumov et al. 2003, Baldwin, Huh et al. 2006), and has been involved in abrogating SRC1-associated HAT activity (Baldwin, Huh et al. 2006).

Both high-risk and low-risk HPV E7 have the ability to interact with the p600 retinoblastoma protein-associated factor (Huh, DeMasi et al. 2005), which is identified as a microtubule-associated protein (MAP) (Shim, Wang et al. 2008) having ubiquitin ligase activity (Tasaki, Mulder et al. 2005). Therefore, the association of HPV-16 E7 with p600 through the CR1 domain may deregulate anoikis and protect detached cells from apoptosis, thus contributing to viral transformation (Gulliver, Herber et al. 1997, DeMasi, Huh et al. 2005, Huh, DeMasi et al. 2005). Recent studies have also identified a novel target of HPV E7, PTPN14, a tyrosine phosphatase and a potential tumour suppressor protein (White, Munger et al. 2016, Szalmas, Tomaic et al. 2017). This protein is subjected to E7-induced, proteasome-mediated degradation through the action of p600 ubiquitin ligase. Over-expression studies showed that PTPN14 decreases the ability of HPV-16 E7 to cooperate with activated EJ-ras in primary cell transformation assays (Szalmas, Tomaic et al. 2017). E7 also associates with centrosomal components, such as Cdk2 and γ -tubulin (Nguyen, Eichwald et al. 2007). The increased Cdk2 kinase activity and interaction of E7 with the centrosome regulator γ -tubulin is associated with centrosome over-duplication and abnormalities which contributes directly towards genome instability and tumour progression (Duensing, Liu et al. 2006). In recent studies, E7 has been shown to play an important role in directly activating the DNA damage response pathway in differentiated human keratinocyte cells and it has been shown that ATM signaling is required for genome amplification (Moody and Laimins 2009, Banerjee, Wang et al. 2011). This is discussed in detail later in the DNA damage response section (Page no. 59-63). Some of the different interaction partners and associated activities of E7 oncoprotein are shown in Figure 5.

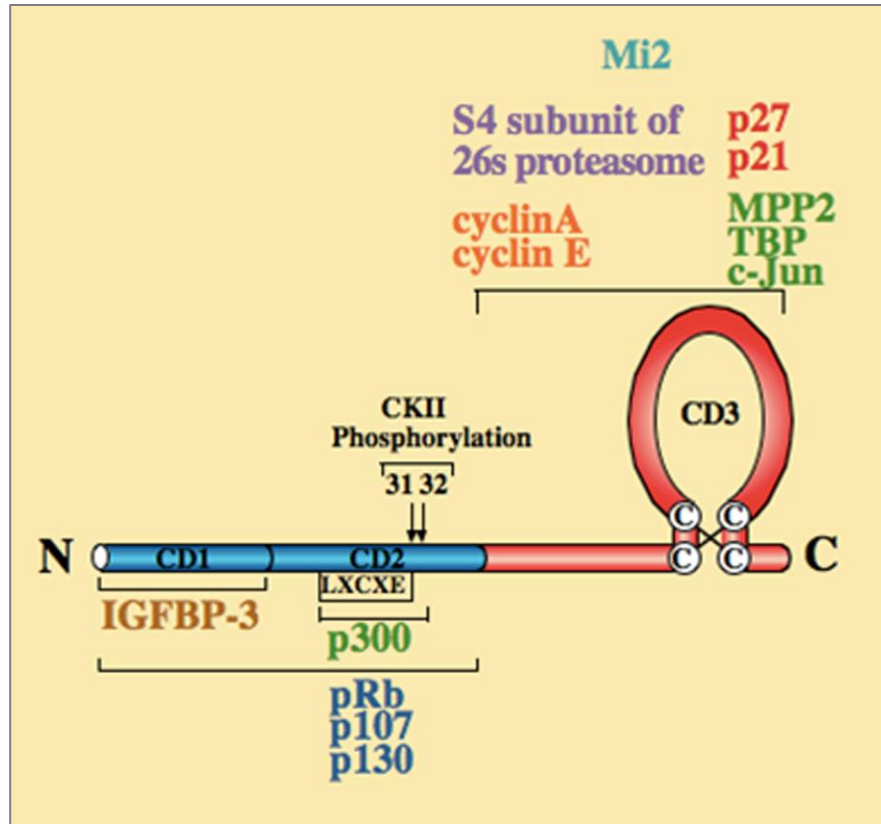


Figure 4. Typical structure of the E7 oncoprotein.

Schematic diagram showing E7 domains: CR1 and CR2 core domain in its N-terminal part and a C-terminal CR3 or Zinc finger domain. The LXCXE motif embedded within the CR2 domain is responsible for interaction with pRB and other pocket proteins; also it contains the region for CKII phosphorylation at S32 and S34 residues.

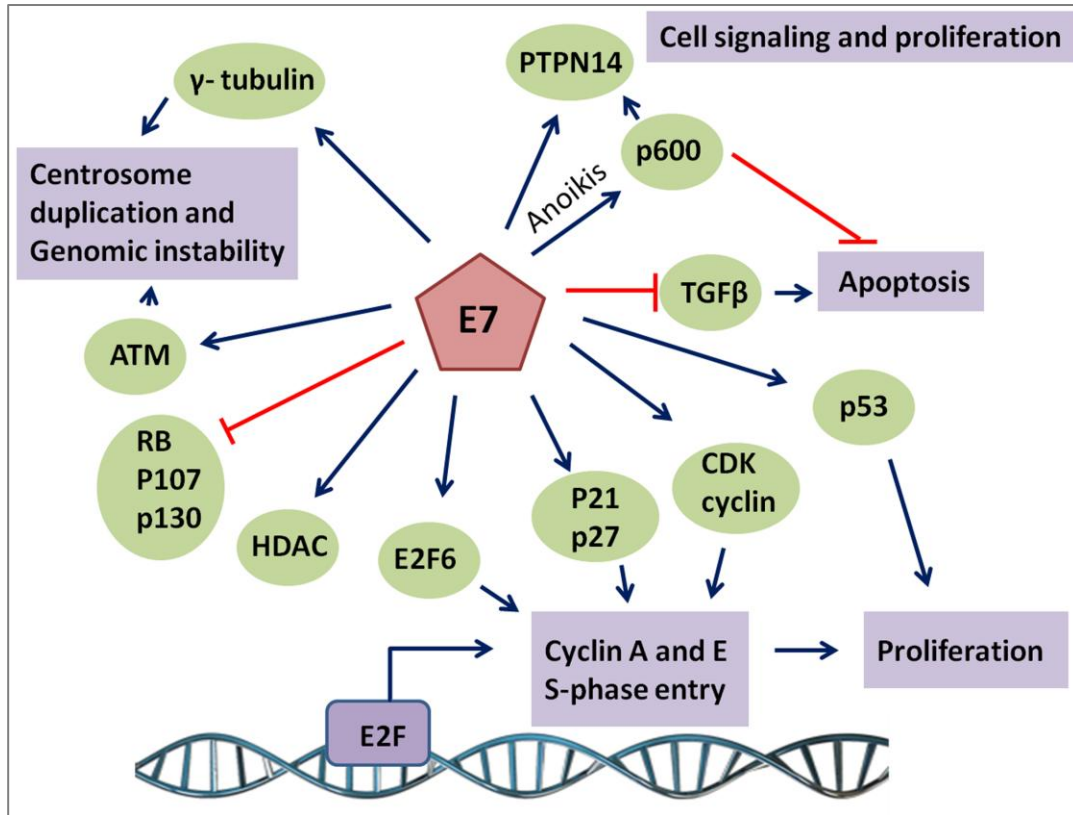


Figure 5. Some of the interacting partners of high-risk HPV E7 and their associated activities.

The E7 association with pRB leads to the proteasome-mediated degradation of pRB and promote S-phase competence in differentiated epithelial cells. Interaction of E7 with p21 and p27 helps in promoting cell proliferation, while the interaction between E7 and HDACs results in increased levels of E2F2-mediated transcription and replication in differentiating cells. Interaction of E7 with p600 potentially affects anoikis, and also targets PTPN14 for degradation. E7 negatively regulates the growth inhibitory activity of TGF- β . E7 directly interacts with and activates ATM signaling, thereby regulating genome replication, while E7's association with γ -tubulin is associated with centrosome overduplication and abnormalities.

HPV E6

HPV E6 is approximately 18kDa in size, and is composed of two 70-residue zinc finger domains (characterized by the presence of zinc-binding CXXC motifs) as shown in Figure 6 (Nomine, Masson et al. 2006). HPV-16 E6 has four mRNA isoforms (full-length (FL)E6, E6*I, E6*II and E6*X) (Tang, Tao et al. 2006), while two mRNA isoforms of E6 are seen in HPV-18 infection (FLE6, E6*I) (Pim, Massimi et al. 1997) and it has been suggested that the E6*I isoform plays an important role in antagonising FLE6 function (Pim, Massimi et al. 1997). HPV-18 E6 is predominantly localized within the membrane fraction, followed by the nucleus and there is a lesser content in the cytoplasm (Kranjec, Tomaic et al. 2016). The NMR and crystallographic analysis of E6 structure shows only 10% sequence identity between the E6N and E6C domains. Both N and C domains of E6 are connected by a linker. The E6C harbors a PDZ-Binding Motif at extream carboxy-terminus in high-risk mucosal HPVs and distinct sequences in other types. (Nomine, Charbonnier et al. 2003, Suarez and Trave 2018). Structural analysis of E6 showed that it is a conserved scaffold with variable interaction surfaces involved in targeting p53 for degradation. E6 can also interact with the E6AP E3 ubiquitin ligase through the E6-binding site present within E6AP, comprising the “LxxLL” motif (Schwarz, Freese et al. 1985, Scheffner, Huibregtse et al. 1993, Talis, Huibregtse et al. 1998, Martinez-Zapien, Ruiz et al. 2016). The E6 protein can self-associate through its N-terminal domain to form a dimer and this has important consequence for the stoichiometry of how different interactions take place (shown in Figure 6). The structural studies show that the homodimer interface mutations that disrupt E6 dimerization can abrogate E6-mediated p53 degradation (Zanier, ould M'hamed ould Sidi et al. 2012, Martinez-Zapien, Ruiz et al. 2016). Unfortunately the structure of E6 PBM in E6-E6AP-p53 complex was not detectable.

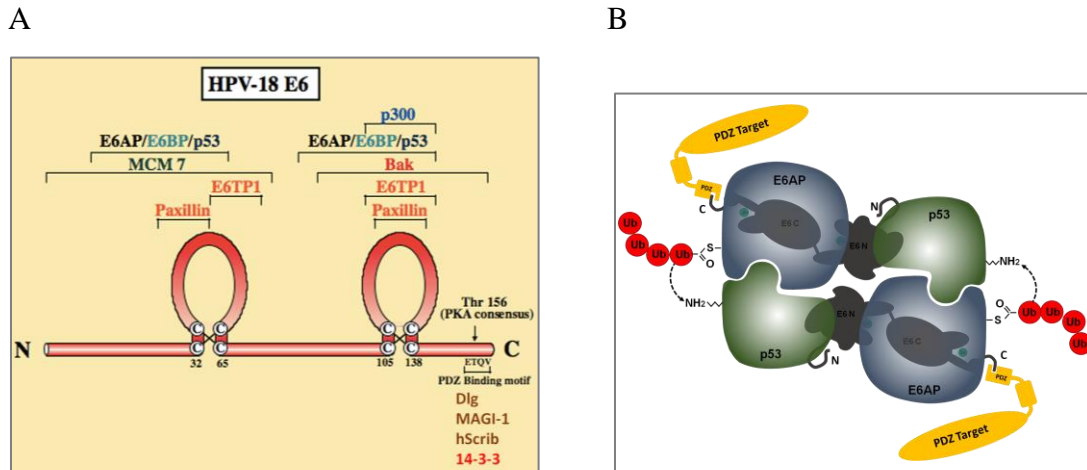


Figure 6. Typical structure of the E6 oncoprotein.

A) Schematic diagram of E6 structure, showing the PBM (PDZ-binding motif) and the phospho-consensus site for PKA and AKT kinases at the C-terminus. Phosphorylation at the PBM confers its association with 14-3-3 family proteins. E6, through the PBM, associates with PDZ domain-containing proteins including hDlg1, MAGI and Scribble. The C-terminal zinc finger interacts with Bak and p300/CBP and targets them for degradation. The two zinc finger motifs create a pocket for the p53 and E6AP interaction, through which E6 targets p53 for proteasomal degradation. B) The schematic model showing a speculative model of E6 dimer, associating with multiple substrates. The E6 PBM is believed to protrude in an unstructured conformation away from the central core of E6 protein. It is speculated that through such complex, E6 can target more than one PDZ domain-containing substrate and also p53 simultaneously.

Some of the key functions of E6

1. Degradation of p53 tumour suppressor

As discussed earlier, HR α -HPV E6 promotes p53 degradation through its association with the cellular E3 ubiquitin ligase, E6AP, preventing p53-mediated cell cycle arrest and apoptosis, and ensuring viral replication. Structural analysis of the E6-E6AP complex reveals that the alpha helix of E6 connects both the N- and C-terminal zinc binding domains to form a deep pocket which could accommodate an LXXLL peptide (Vande Pol and Klingelutz 2013, Martinez-Zapien, Ruiz et al. 2016). This interaction is thought to be of great importance for E6 stability (Tomaic, Pim et al. 2009). The formation of the complex between E6-E6AP-p53 requires homodimerization of E6 at its N-terminus (Zanier,ould M'hamed ould Sidi et al. 2012), and the ability of E6 to target p53 for degradation is crucially dependant on the formation of this complex as described and illustrated earlier in figure 6B. E6AP transfers ubiquitin from its C-terminal thioester cysteine bond to p53 which is then targeted for degradation (Scheffner, Huibregtse et al. 1993). Ablation of E6AP rescues p53 levels, both as a result of E6 destabilization and the loss of ubiquitin ligase activity (Hengstermann, D'Silva M et al. 2005, Tomaic, Pim et al. 2009). Interestingly, HPV E6 can also induce auto-ubiquitination of E6AP. The half-life of E6AP is shorter in HPV-positive cervical cancer cells than in HPV-negative cervical cancer cells and silencing of E6 can increase the levels of E6AP protein. The degradation of E6AP by E6 requires: i) the binding of E6 to E6AP ii) the catalytic activity of E6AP and iii) the 26S proteasome. Importantly, a study by Kao *et al.* demonstrates that, an HPV-16E6 SAT8–10 mutant which is unable to degrade p53, but can target E6AP for degradation, can still immortalize human mammary epithelial cells. This suggests that interaction with E6AP *per se* is an important factor for the transforming activity of the high-risk HPV E6 proteins (Kao, Beaudenon et al. 2000). Indeed, studies in animal models show a critical requirement of E6AP for E6's full transforming ability. However, it remains to be determined how much is

due to the effect of E6AP on E6 stability and how much related to E6AP's enzymatic activity, which is utilized by E6 to degrade its substrates.

2. Degradation-independent p53 inactivation

HR α -HPV E6 indirectly regulates the functions of p53 by altering the activation of p53-modifying enzymes. In response to DNA damage, ATR is activated, which further phosphorylates p53, thus blocking MDM2-mediated degradation of p53 (Tibbetts, Brumbaugh et al. 1999). Recent studies suggest that E6 perturbs p53-dependent transcriptional transactivation after the onset of the DNA damage response, by delaying ATR activation (Wallace, Robinson et al. 2012, White, Walther et al. 2014). p300, a histone acetyltransferase, acetylates p53, thus increasing its binding specificity and upregulating the transcription of its target genes (Gu and Roeder 1997). E6 interacts with CBP/p300 through its C terminal CXXC motif and this interaction is thought to inhibit p53 transcriptional transactivation and to inhibit transactivation of the p300-induced, pro-apoptotic nuclear NF- κ B. Moreover, E6-interacting regions of p300 are important for the ability of E6 to inhibit p53-dependent chromatin transcription, and this correlates with inhibition of acetylation on p53 and nucleosomal core histones, thus, altering recruitment of p53 and p300 to chromatin. (Patel, Huang et al. 1999, Zimmermann, Degenkolbe et al. 1999, Thomas and Chiang 2005). E6 also targets other histone acetyltransferases, including hADA3 and TIP60 that also affect p53 transcriptional transactivation. E6 inhibits the activity of transcriptional coactivator, hADA3 by proteasome-mediated degradation which is an alternative mechanism for inhibiting p53 transcriptional transactivation (Kumar, Zhao et al. 2002, Sekaric, Shamanin et al. 2007, Shamanin, Sekaric et al. 2008, Chand, John et al. 2014). Similarly, E6 modulates the functions of the TIP60 histone acetyltransferase, wherein, TIP60 is destabilized and subsequently p53-dependent transcription of pro-apoptotic genes is perturbed (Jha, Vande Pol et al. 2010).

The region of p53-E6 association appears to be different between the HR and the LR HPV types. However, studies show that p53-E6 binding is necessary, but not sufficient for the ability of E6 to target p53 for degradation (Crook, Tidy et al. 1991). The low-risk E6 binds to the C-terminal region of p53, whilst high-risk E6 has two distinct binding sites within p53, of which only one is responsible for p53 degradation. (Li and Coffino 1996). Studies on HPV 11 show that low-risk E6 can interact with p53 through E6AP *in vivo* but not *in vitro*, and this interaction does not mediate p53 degradation, unlike high risk E6s (Brimer, Lyons et al. 2007, White, Kramer et al. 2012, Thomas, Tomaic et al. 2013). However, under certain circumstances, the low risk HPV-11 E6 oncoprotein has been reported to degrade p53 (Storey, Thomas et al. 1998). Furthermore, low risk HPV E6 proteins can inhibit p53 transcriptional transactivation (Kiyono, Hiraiwa et al. 1994) by repressing the p53 transcription of TATA box-containing promoters (Lechner, Mack et al. 1992), and also by maintaining p53 in the cytoplasm (Sun, Zhang et al. 2008).

3. Telomerase activation, transformation and immortalization

Another important function of E6 is to activate telomerase by a mechanism independent of p53 degradation, and this step is crucial for immortalization (Klingelutz, Barber et al. 1994, Klingelutz, Foster et al. 1996). E6 is involved in increasing hTERT activity, either through direct interaction or through interaction with Myc, thereby up-regulating E-box-dependent transcription of hTERT for immortalization (Oh, Kyo et al. 2001). E6 can cooperate with NFX1-123 to increase hTERT activity (Vliet-Gregg, Hamilton et al. 2013) and down-regulate the hTERT repressors, such as NFX1-91 (Gewin, Myers et al. 2004), p300 (James, Lee et al. 2006) and Maz (Xu, Katzenellenbogen et al. 2013). (Klingelutz, Barber et al. 1994, Klingelutz, Foster et al. 1996). The advantage of activation of telomerase by HPV is not entirely clear. One of the possible advantages could be manipulation of DNA damage pathway for its own replication, as telomeres are associated with a variety of DDR proteins (Wallace and Galloway 2014).

4. Abrogation of apoptotic signaling

One of the peculiar characteristics of E6 is to interact with cellular pro- and anti-apoptotic proteins to prevent the activation of growth inhibitory pathways (Ishiwatari, Hayasaka et al. 1994, Nakagawa, Watanabe et al. 1995). E6 proteins can inhibit apoptosis in both p53-dependent and p53-independent manners. Both high risk and low risk E6s can interact and degrade the pro-apoptotic protein Bak through cooperation with the E6AP ubiquitin ligase (Thomas and Banks 1998, Thomas and Banks 1999). This activity is believed to be important for many HPV types, as it appears that cutaneous HPV types can also inhibit apoptosis in response to UV-induced DNA damage in a manner which is dependent upon Bak degradation (Jackson, Harwood et al. 2000, Jackson and Storey 2000). E6 also modulates the functions of anti-apoptotic Bcl-2 and pro-apoptotic Bax, thereby suppressing apoptosis in human foreskin keratinocytes during serum-calcium switch-induced differentiation. In the presence of E6, the expression levels of Bcl-2 are increased, whereas Bax levels are decreased, thus preventing apoptosis (Alfandari, Shnitman Magal et al. 1999).

Additionally, it has been shown that the RNAi-targeted inhibition of E6 expression in cervical cancer cells leads to the transcriptional stimulation of the PUMA promoter, in a p53-dependent manner and this is linked to the PUMA-dependent activation and translocation of Bax to the mitochondrial membrane, subsequent release of cytochrome c into the cytosol, and activation of caspase-3. Moreover, inhibition of Bax expression by RNAi efficiently reverts the apoptotic phenotype, which results from inhibition of E6 expression. Thus, interference with the p53/PUMA/Bax cascade is crucial for the antiapoptotic function of the viral E6 oncogene in HPV-positive cancer cells (Vogt, Butz et al. 2006). E6 also manipulates Fas and caspase signaling by decreasing the stability and activity of Fas-associated death domain (FADD) and procaspase 8 in the cells. The full length E6 can directly interact with FADD and target it for degradation which prevents transmission of apoptotic signals via the Fas pathway (Filippova, Parkhurst et al. 2004, Filippova, Johnson et al. 2007). E6 also downregulates the expression of the pro-apoptotic

protein transforming growth factor- β 2 (TGF- β 2) and thus downregulates TGF- β 2 responsive genes (Nees, Geoghegan et al. 2000).

5. Attenuation of cell-signaling

The E6 protein targets many major cellular signaling pathways, including the NF- κ B-TNF, mammalian target of rapamycin (mTOR)-AKT, MAPK, and Notch pathways. The association of HPV E6 with TNF R1 interferes with the formation of the death-inducing signaling complex. It abrogates transduction of proapoptotic signals, resulting in reduced Caspase 3 and caspase 8 activation in E6-expressing cells (Filippova, Song et al. 2002). E6 is involved in activation of mTOR-AKT, which are serine/threonine kinases in the PI3K pathway (Spangle and Munger 2010) and E6 achieves this by degradation of the mTOR inhibitor, tuberous sclerosis complex 2 (TSC2) through E6AP (Lu, Hu et al. 2004, Zheng, Ding et al. 2008). This leads to activation of NF- κ B (Basseres and Baldwin 2006) and resistance to TNF receptor-induced apoptosis (Dolcet, Llobet et al. 2005).

The expression of HPV-16 E6, prevents the early fate commitment of human keratinocytes towards differentiation and confers a strong growth advantage to human keratinocytes. When E6 is expressed either alone or with E7, it induces combined inactivation of p53 and Notch1, thus promoting keratinocyte proliferation at high cell densities (Kranjec, Holleywood et al. 2017). Studies in cutaneous papillomavirus E6 oncoproteins show that, their association with MAML1, a co-activator and effector of Notch-induced transcription, represses Notch signaling, thereby delaying keratinocyte differentiation (Brimer, Lyons et al. 2012). Additionally, studies have shown that the E6 mediated activation of MAPK signaling cooperates with deregulated Notch1 signaling to recreate features of HPV-driven invasive cervical carcinomas (Chakrabarti, Veeraraghavalu et al. 2004). Recent study shows that Foxhead box M1 (FOXM1) expression is upregulated by E6 in HPV-positive cervical, oral, and lung cancer cells and this is linked to increased invasiveness and stemness through activating Wnt/ β -catenin signaling pathway (Chen, Cheng et al. 2014).

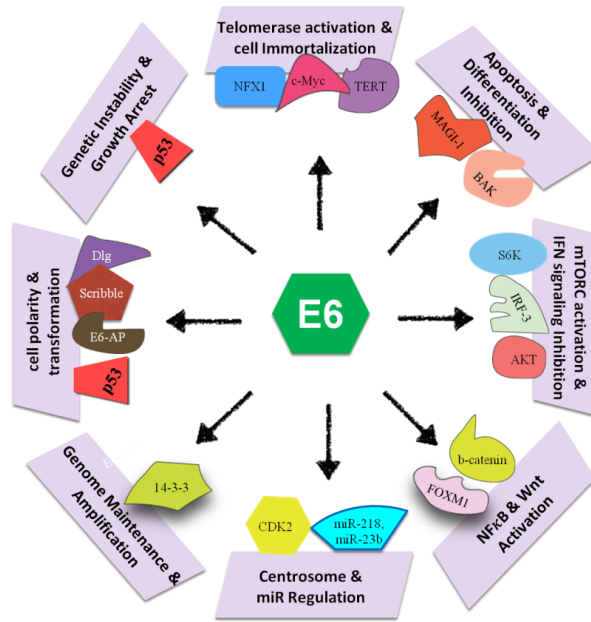


Figure 7. Some of the high-risk HPV E6 interacting partners and associated activities.

E6 interacts with c-Myc and NFX-1 to induce the hTERT promoter, contributing toward cellular immortalization. E6 hijacks E6AP ubiquitin ligase, which is essential for E6 stability and for targeting p53 for proteasome-mediated degradation, thus resulting in genome instability and inhibiting growth arrest. The E6, through its PBM, interacts with a large number of cellular PDZ domain-containing substrates, such as Dlg and Scribble, which are cell polarity proteins and their interaction is believed to affect malignant progression. On the other hand, phosphorylation of the PBM confers its interaction with 14-3-3 proteins, which are important cell cycle-regulatory proteins. The interaction of E6 with MAGI-1 regulates cell-cell contact, proliferation, and apoptosis. E6 can stimulate mTORC1 signaling and can inhibit interferon signaling through its interaction with AKT. E6 associates with BAK to promote its degradation, thus preventing the induction of apoptosis. E6 regulates NFκB functions and induces Foxhead box M1 (FOXM1)-mediated tumor progression. E6 also regulates the Wnt/β-catenin signaling, thereby affecting cell proliferation and differentiation. E6 affects the expression of miR 218 and miR23b, and in addition, its interaction with CDK2 can affect cell cycle regulation.

The E6 PDZ binding motif (PBM): Molecular signature of cancer-causing HPV types

The high- and low-risk HPV types exhibit major structural differences within the E6 C-terminal region. The presence of a PDZ (PSD-95/DLG/ZO-1) binding motif (PBM) (Songyang, Fanning et al. 1997) in high-risk HPV types correlates with their oncogenic potential and this motif is absent in the majority of the low-risk HPV types (Kiyono, Hiraiwa et al. 1997, Lee, Weiss et al. 1997, Songyang, Fanning et al. 1997). The high-risk HPV E6 oncoproteins, possess the canonical X-S/T-X-COOH PBM sequence, although different HPV E6 types display significant degrees of variation within this region, as can be seen in Figure 8. PDZ domains are 80 to 100 amino acids in length and facilitate protein-protein interactions via ligands containing a PBM. Multiple copies of these PDZ domains are often found in proteins that are involved in multiple protein interactions, often acting as hubs for a variety of different signaling pathways (Fanning and Anderson 1999). Proteins with PDZ domains play crucial roles in regulating cell migration and invasion, in maintaining cell polarity and cell-cell contact, and in cell signaling (Jacob, Oppen et al. 1987, Woods and Bryant 1991, Woods, Hough et al. 1996, Bilder, Li et al. 2000, Bilder 2004, Subbaiah, Kranjec et al. 2011). Several studies have shown that many of these proteins play important roles as potential tumour suppressors (Javier 2008). E6 interacts with multiple PDZ-containing proteins and targets many of them, such as hDlg (Kiyono, Hiraiwa et al. 1997, Lee, Weiss et al. 1997), the Scribble (Nakagawa and Huibregtse 2000) and MAGI proteins (Glaunsinger, Lee et al. 2000) for proteasome-dependent degradation.

Regulation of cell polarity and cell adhesion

The first PDZ protein that was identified as a target of HR HPV E6 is Discs large (hDlg) (Kiyono, Hiraiwa et al. 1997, Lee, Weiss et al. 1997). hDlg is a member of the membrane-associated guanylate kinase (MAGUK) family (Gonzalez-Mariscal,

Betanzos et al. 2000), which are found at junctions between epithelial cells, where they maintain the integrity of the cell junctions, the localisation of basolateral membrane proteins and apico-basal cell polarity (Woods and Bryant 1991, Woods and Bryant 1993, Woods, Hough et al. 1996). They also act as scaffold proteins, and are thus involved in signal transduction from the plasma membrane to the downstream regulators of the cell polarity pathway and control cell proliferation (Anderson 1996). Mislocalisation or even loss of expression of the cell junctional PDZ protein complexes often results in loss of cell polarity and can contribute to carcinogenesis (Gateff and Schneiderman 1974, Mechler, McGinnis et al. 1985, Bilder, Li et al. 2000).

E6 targets hDlg for degradation, thus contributing to a loss of cell polarity, morphological transformation and proliferation. However, the ability of E6 to target hDlg can be influenced both by its phosphorylation status and also its location (Matsumine, Ogai et al. 1996, Gaudet, Branton et al. 2000, Narayan, Massimi et al. 2009). Studies have demonstrated that the interaction of HR HPV types with hDlg protein is well conserved (Gardiol, Kuhne et al. 1999, Pim, Thomas et al. 2000).

Scribble (hScrib) belongs to cell polarity complex and is a member of the LAP (leucine-rich PDZ domain) family of proteins; it contains 4 PDZ domains and a leucine-rich region which is essential for its basolateral localisation (Bryant and Huwe 2000, Santoni, Pontarotti et al. 2002, Legouis, Jaulin-Bastard et al. 2003). Loss of hScrib expression results in changes of cell shape, loss of monolayer organisation and loss of cell-cell junctions (Bilder, Li et al. 2000, Bilder and Perrimon 2000, Zhan, Rosenberg et al. 2008). E6 degrades hScrib through the E6AP-ubiquitin ligase (Nakagawa and Huibregtse 2000, Thomas, Massimi et al. 2005), and HPV-16 E6's interaction with hScrib is more effective than HPV-18 E6's (Thomas, Massimi et al. 2005). The reason behind this is a minor difference in the amino acid sequence of the PBM between different E6 oncoproteins. Indeed, it has been shown that swapping the last amino acid L/V of HPV-16 and -18 E6,

respectively, reverses the preference for hDlg or hScrib proteins (Thomas, Massimi et al. 2005).

Membrane-associated guanylate kinase (MAGI-1) is present at tight junctions, and is involved in regulating cell proliferation, apoptosis and cell signaling. MAGI-1 was originally found co-localised with one of the important components of the epithelial tight junctions, zonula occludens-1 (ZO-1) (Dobrosotskaya, Guy et al. 1997). The PDZ domain 1 of MAGI-1 protein is recognized by HPV E6 (Glaunsinger, Lee et al. 2000), and E6 then targets it for degradation, thus leading to the disruption of tight junctions (Kranjec and Banks 2011, Kranjec, Massimi et al. 2014). Restoration of MAGI-1 expression in HPV-positive cell lines allows the tight junction proteins ZO-1 and PAR-3 to localize at the cell-cell contact area (Kranjec, Massimi et al. 2014). In addition to MAGI-1, the related proteins MAGI-2 and MAGI-3 are also targeted by HR HPV E6 for degradation (Thomas, Laura et al. 2002).

Recent studies highlight novel function of high-risk HPV E6 oncoproteins, directly regulating endocytic transport pathways. This activity is carried out through interaction of the E6 PBM with endocytic cargo sorting machinery, via sorting nexin 27 (SNX27). This interaction directly impacts upon rates of cargo recycling, and as a consequence of this, HPV-transformed cells show high levels of glucose uptake (Ganti, Massimi et al. 2016). This is one example of the E6-PDZ interactions that do not result in degradation of the PDZ domain-containing target protein, rather in the modulation of its function.

Importance of the E6 PBM

Numerous studies have shown an important role for the E6 PBM. In the viral life cycle, it is essential for episomal maintenance and genome amplification during a productive viral infection (Brimer and Vande Pol 2014). An intact E6 PBM is crucial for maintaining the viral copy number in undifferentiated cells (Lee and Laimins

2004, Delury, Marsh et al. 2013). It has also been reported to play an important role in the ability of E6 to induce cell transformation in certain cell types, and to play a role in the induction of epithelial to mesenchymal transition (Spanos, Geiger et al. 2008). Studies on HPV-18 genomes in HFK cells have shown that cells which harbor E6 Δ PBM genomes, proliferate somewhat more slowly than the cells containing wild type genomes.

Other studies in HFKs showed that the PBM of E6 is essential for induction of epithelial hyperplasia in raft cultures, and also that it contributes to the maintenance of viral episomes (Lee and Laimins 2004, Delury, Marsh et al. 2013, Choi, Lee et al. 2014). Moreover, the PBM has been shown to play essential roles, not only in genome maintenance, but also in creating a favorable environment for viral replication; as extended culturing/passaging of cells resulted in loss of E6 Δ PBM genomes (Nicolaidis, Davy et al. 2011). A number of studies in Normal Immortal keratinocytes (NIKS) harboring HPV-16 genomes lacking E6 PBM also showed defects in the viral life cycle. However, in this particular case, it was potentially due to the loss of E6-PDZ interactions and to reduced levels of E6 protein expression (Nicolaidis, Davy et al. 2011). However, this does not appear to be as a result of E6/PDZ interaction, since loss of hScrib results in a decrease in the translation of the E6, indicating that there are yet unknown signaling pathways downstream of hScrib that directly impact upon protein translation (Kranjec, Tomaic et al. 2016).

In animal models of skin and cervical cancer, the E6 PBM also appears to play a major role in the induction of epithelial hyperplasia and acts in cooperation with E7 for the induction of malignancy (Nguyen, Nguyen et al. 2003, Shai, Brake et al. 2007). However, which of the PDZ substrates are involved in regulating these activities of the E6 PBM is still largely unknown.

Phospho-regulation of E6

E6 also has a phospho-acceptor site embedded within its PBM and thus E6 can potentially be subject to post-translational modification by phosphorylation. Post-translational modification plays a major role in regulating E6 function. As noted above, E6/Dlg recognition can be modulated by Cdk phosphorylation of Dlg (Narayan, Massimi et al. 2009, Boon and Banks 2013). However, a more specific mode of regulation is provided by phosphorylation of E6 within the PBM. Central to the consensus PBM is a S/T at the -2 position in all the high-risk HPV E6 oncoproteins. This can often be phosphorylated either by PKA or AKT kinases. The net result thereby is inhibition of PDZ recognition. This phenomenon is generally recognized as effective means of regulating PBM/PDZ interactions, since the phosphorylation of Threonine within the PBM is incompatible with PDZ domain recognition (Kuhne, Gardiol et al. 2000). The molecular basis for this is well understood and reported for many PBM-PDZ ligand combinations. Many studies demonstrate that the presence of phospho group is incompatible with fitting into the limited space of the PDZ domain-PBM binding pocket.

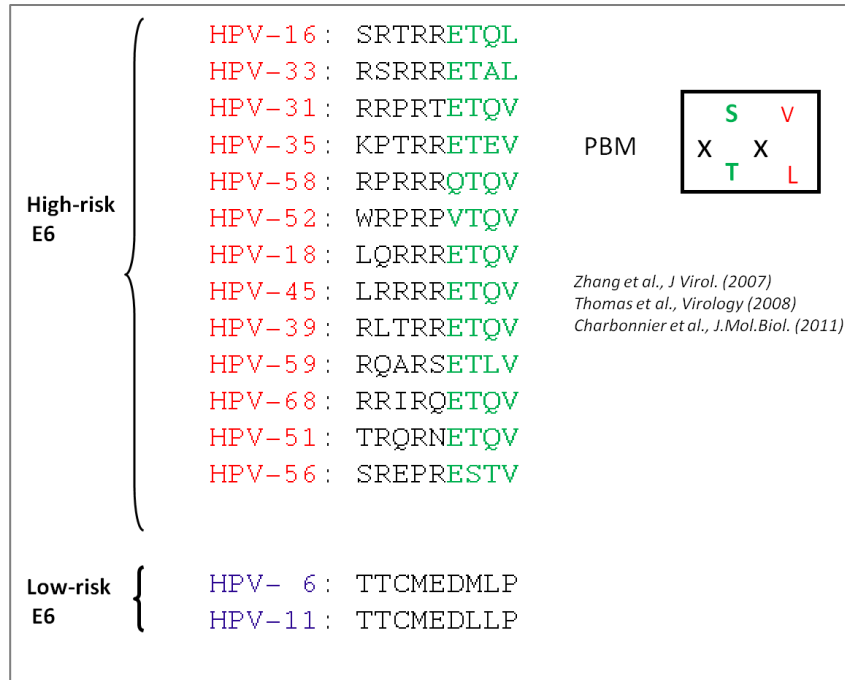


Figure 8. Sequences of E6 PBMs.

A representative series of different high-risk HPV types shows the presence of conserved PBM sequence (x-T/S-x) at the C-terminus (Shown in green). This motif is absent in low risk types. Also shown is the sequence upstream of phospho-acceptor site. This sequence plays important role in E6-substrate selection.

14-3-3: important cell cycle regulatory proteins

14-3-3 proteins are highly acidic proteins, with molecular weights of 25 to 33 kDa (Jones, Ley et al. 1995) and they are abundantly present in cells. There are seven isoforms of 14-3-3: α/β , γ , ϵ , ζ/δ , η , τ/θ and σ (Mackintosh 2004), and they exist as homo- or hetero-dimers with other 14-3-3 isoforms (Jones, Ley et al. 1995), and expression pattern of 14-3-3 isoforms differs in different tissue types (Yaffe, Rittinger et al. 1997, Kilani, Medina et al. 2008). The specificity of 14-3-3 function is conferred by dimerization, and only a certain combinations of 14-3-3 isoforms form dimers. The 14-3-3 proteins are phospho-threonine/serine binding proteins (Muslin, Tanner et al. 1996) and they bind to target proteins with specific phosphothreonine and phosphoserine motifs and alter their intercellular localization and activity (Muslin, Tanner et al. 1996). These proteins regulate many different cellular processes, as listed in Table 1, including cell cycle progression, intracellular protein trafficking, apoptosis, DNA damage response, DNA replication, and transcriptional regulation. The 14-3-3 ϵ , γ , and ζ isoforms have been shown to activate Raf, PI3K, and MSK1/2 for regulation of the ERK signaling pathway in variety of cell types (Lin, Morrison et al. 2009, Neal and Yu 2010). Overexpression of 14-3-3 ζ has been implicated in promoting cancer cell proliferation, whereas the downregulation of 14-3-3 ζ inhibits cell proliferation by inducing the mitochondria-dependent apoptosis pathway (Chatterjee, Goldman et al. 2004, Li, Zhao et al. 2008, Maxwell, Cherry et al. 2011). Studies in a mouse models demonstrated that inhibition of 14-3-3, either by difopein, a general 14-3-3 antagonist, or by 14-3-3 siRNA, induced apoptosis in human glioma cells and suppressed the growth of the tumour (Cao, Yang et al. 2010). 14-3-3 binding has been shown to protect the target protein from other modifications, such as dephosphorylation (Chen and Wagner 1994, Dent, Jelinek et al. 1995, Jelinek, Dent et al. 1996, Chiang, Harris et al. 2001) and proteolysis (Weiner and Kaiser 1999, Cotelle, Meek et al. 2000). Interaction of 14-3-3 with certain targets can alter the ability of the target protein to interact with its partners. For instance, in the case of BAD, 14-3-3 binding

competes with the BAD-Bcl2 association, releasing Bcl2 to perform its anti-apoptotic function (Zha, Harada et al. 1996, Hsu, Kaipia et al. 1997, Datta, Katsov et al. 2000). In the case of IRS-1 and PI-3 kinase, 14-3-3 binding to IRS-1 attenuates its ability to recruit and activate PI-3 kinase (Kosaki, Yamada et al. 1998).

14-3-3 functions in cell cycle checkpoints

When cells encounter cell cycle checkpoint activation in response to DNA damage, Cdc25 is phosphorylated by TAK or Chk1/Chk2 at S216 (Peng, Graves et al. 1997, Sanchez, Wong et al. 1997, Peng, Graves et al. 1998, Liu, Guntuku et al. 2000), and this generates a 14-3-3 binding motif. As a consequence of this phosphorylation, Cdc25B and Cdc25C are sequestered in inactive forms in the cytoplasm by 14-3-3 (Peng, Graves et al. 1997, Forrest and Gabrielli 2001, Giles, Forrest et al. 2003, Uchida, Kuma et al. 2004, Esmenjaud-Mailhat, Lobjois et al. 2007, Astuti, Boutros et al. 2010, Chan, Ng et al. 2011), and cannot then dephosphorylate the Cdk1/Cyclin B complex thus halting the cell cycle progression into mitosis (Dalal, Yaffe et al. 2004, Uchida, Kuma et al. 2004, Hermeking and Benzinger 2006, Esmenjaud-Mailhat, Lobjois et al. 2007).

In the G1/S phase, Cdc25A is phosphorylated by Chk1 (Sanchez, Wong et al. 1997, Jin, Shirogane et al. 2003) and this allows the interaction of Cdc25A with 14-3-3 γ in a phosphorylation-dependent manner, which ultimately leads to sequestration of Cdc25A in the cytosol, triggering its degradation (Chen, Ryan et al. 2003, Jin, Shirogane et al. 2003, Kang, Wei et al. 2008, Kasahara, Goto et al. 2010). 14-3-3 σ can associate directly with Cdk2 and Cdk4, and thereby prevent cell cycle entry by inhibiting cyclin-Cdk function (Laronga, Yang et al. 2000).

14-3-3 can associate with phosphorylated p53, shifting the dimer-tetramer equilibrium of p53 to the DNA-binding tetrameric form, and leading to form a high affinity complex with DNA (Waterman, Stavridi et al. 1998, Rajagopalan, Jaulent et al. 2008). Recent studies have shown that phosphorylation of p53 at S366 or T387

leads to its association with 14-3-3 γ and ϵ , and that this interaction enhances p53's DNA binding ability 10-fold, thereby enhancing p21 transcriptional activation (Rajagopalan, Jaulent et al. 2008).

Additionally, phosphorylation of p27 by AKT at T157 confers its interaction with 14-3-3 β , ϵ , γ , ζ and θ , whilst phosphorylation of p27 at T198 confers interaction with 14-3-3 ϵ , η and τ , but not β and ζ , thus retaining p27 in the cytosol (Fujita, Sato et al. 2002, Shin, Yakes et al. 2002, Viglietto, Motti et al. 2002, Sekimoto, Fukumoto et al. 2004, Kossatz, Vervoorts et al. 2006, Short, Dere et al. 2010).

Cellular process	Target	Sequel
14-3-3 γ , η , ζ/δ and ϵ	Phosphorylation of Cdc25	Regulation of CHK 1 kinase
14-3-3 η , ζ/δ and ϵ	Wee 1, CDK1	Cell cycle regulation
14-3-3 γ , ϵ , σ and τ/θ	p53 activation	Cell cycle regulation via ATM/ATR activation

Table 1. Different 14-3-3 isoforms and their respective functions during the cell cycle.

Phospho-regulation of 14-3-3

14-3-3 proteins themselves are regulated by phosphorylation, which in turn can affect 14-3-3 dimerisation. 14-3-3 can be phosphorylated at residues S58, S59, S64, S185 and S233 by several different kinases (Dubois, Howell et al. 1997). PKA phosphorylation of 14-3-3 ϵ at S58 has been shown to affect its dimerisation and affects its interaction with p53 (Gu, Jin et al. 2006). Likewise, phosphorylation of 14-3-3 ζ at S58 by MAPK-activated protein kinase 2 inhibits the dimerisation of 14-3-3 and its association with Raf-1 (Powell, Rane et al. 2003). Additionally, DNA damage-dependent association of phosphorylated Chk1 with 14-3-3 proteins mediates an important step along the DNA-damage checkpoint pathway, perhaps by

directing Chk1 to a particular substrate or to a particular location within the cell (Chen, Liu et al. 1999). The stimulated association of Chk1 with 14-3-3 proteins is compromised in mutants defective for the DNA-damage checkpoint pathway suggesting that their interaction is critically dependent on DDR. This also highlights the regulation of phosphorylation dependent 14-3-3 associated activities in DDR pathway.

A link between E6 PBM-14-3-3 and p53

Interestingly, a number of recent studies with HPV-16 and HPV-18 genomes in NIKS cell lines have shown that the E6 PBM is required for episomal maintenance of the two viral genomes. Studies with HPV-16 genomes demonstrated that wild-type HPV-16 replicated episomally in NIKS, NIKS-p53_shRNA, and NIKS-p53_GSE56 cells. HPV-16_E6 Δ PBM (deletion of last two amino acids) was not maintained in NIKS cells but was maintained as an episomal plasmid in both NIKS_GSE56 and NIKS_p53_shRNA cells after 8 weeks of cell culture. (Lorenz, Rivera Cardona et al. 2013, Brimer and Vande Pol 2014). This suggests that the E6 PBM might contribute towards perturbation of p53 functions, independently of E6's ability to degrade p53. One potential common link between these observations is 14-3-3, since this family of proteins is linked to the regulation of p53 transcriptional transactivation activity during cell cycle check point control (Waterman, Shenk et al. 1995, Waterman, Stavridi et al. 1998, Rajagopalan, Jaulent et al. 2008, Rajagopalan, Sade et al. 2010). As noted above 14-3-3 interacts with p53 in a phosphorylation-dependent manner (Agarwal, Agarwal et al. 1995, Waterman et al, 1998). Following induction of DNA damage, S376 of p53 is dephosphorylated, while S378 and T387 are phosphorylated by Chk1 and Chk2 kinases. This allows binding of 14-3-3 γ , ϵ , θ and σ proteins with p53. This in turn increases the affinity of p53 binding to DNA and subsequent activation of p53 responsive promoters such as p21.(Agarwal, Agarwal et al. 1995, Waterman, Stavridi et al. 1998, Rajagopalan, Jaulent et al. 2008). Taken together, these findings suggest that there could be potential links

between E6 PBM phosphorylation and p53 transcriptional inactivation through its association with 14-3-3.

Ubiquitin-Protein Ligases

Ubiquitination is an important post-translational modification process that is accomplished via a catalytic cascade involving an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub-protein ligase (E3) (Hershko and Ciechanover 1998, Eletr, Huang et al. 2005, Lorenz, Cantor et al. 2013, Olsen and Lima 2013). Based on the differences in their ubiquitination mechanisms, E3 Ub-protein ligases are classified into two groups: RING-type and HECT-type. RING (Really Interesting New Gene) E3s add Ub directly from E2 to the substrate, functioning as a scaffold (Zheng, Wang et al. 2000), as shown in Figure 9 whereas, HECT-type E3s add Ub from E2 to the substrate via the catalytic cysteine of the HECT domain (Wang and Pickart 2005, Rotin and Kumar 2009). HECT-type E3s have a HECT (homologous to E6AP carboxyl-terminus) domain and a substrate recognition site at the N-terminus and the HECT- domain is highly conserved between all HECT-type E3s. This domain, as the name suggests, shows homology with the C-terminal region of E6AP (E6-associated protein) and it is composed of about 350 amino acids (Purbeck, Eletr et al. 2010).

Various HECT-domain E3 ligases have been shown to be involved in a number of diseases and cancers. They include: E6AP, EDD, Nedd4-1, Nedd4-2, HERC1, HERC2, HERC5, Smurf1, Smurf2, Itch, and HETCH9. The E6AP ligase is known to be involved in Angleman syndrome (Albrecht, Sutcliffe et al. 1997, Rougeulle, Glatt et al. 1997, Vu and Hoffman 1997), in cervical cancer (Talis, Huibregtse et al. 1998), and is also suggested to be associated with autism (Yi, Berrios et al. 2015). EDD has been shown to be overexpressed in ovarian cancers (Bradley, Zheng et al. 2014). Itch was found to be associated with lung and stomach inflammation and in lymphoid and

hematopoietic hyperplasia (Fang, Elly et al. 2002). Overexpression of Smurf1 and Smurf2 has been found to be associated with pancreatic and esophageal squamous cell carcinomas (Fukuchi, Fukai et al. 2002, Loukopoulos, Shibata et al. 2007), while Nedd4-1 and Nedd4-2 are found associated with Liddle's syndrome (Staub, Dho et al. 1996),(Reviewed in (Scheffner and Staub 2007).

Ubiquitination of target proteins is achieved by the generation of an isopeptide bond between the carboxy group of the C-terminal Gly76 residue of Ub and the 3-amino group of Lysine residues in the substrate proteins (Rotin and Kumar 2009). Ubiquitination is a multistep ATP-dependent process. In the first step, a thioester bond is formed between the C-terminus of ubiquitin and an internal Cys residue of the E1. The activated ubiquitin is then transferred to a specific Cys residue of the E2 enzyme. In the next steps, E2 either donates ubiquitin directly to the target protein through E3 ubiquitin ligase activity, or through specific HECT E3s. This process gives rise to protein conjugates, in which the C-terminus of ubiquitin is linked by an isopeptide bond to specific internal Lys residues of the substrates. Substrates can be modified by E3s by either adding one single ubiquitin molecule (monoubiquitylation), adding several single ubiquitin molecules at multiple Lys residues (multiubiquitylation), or adding multiple ubiquitin molecules to a single site, forming a polyubiquitin chain (polyubiquitylation) (Hoeller, Hecker et al. 2007).

The fate of ubiquitylated proteins is dependent on the type of ubiquitin chain present on the substrate, and also on the type of isopeptide linkage formed by the polyubiquitin chain. Thus, different types of Ub modifications have distinct functions in the cell, for example: Poly-Ub chains linked through the Lys-48 of Ub label the protein for proteasomal degradation, whereas poly-Ub chains linked through Lys-63 are involved in endocytosis and DNA repair. Monoubiquitination is also connected with endocytosis and DNA repair, whilst multiubiquitination is linked to endosomal sorting and lysosomal degradation (Pickart and Fushman 2004, Haglund and Dikic 2005). The majority of Lys-48-linked polyubiquitylated proteins

are degraded by the 26S proteasome, while the ubiquitin moieties are recycled following their release by deubiquitylating enzymes (Bernassola, Karin et al. 2008). In addition to Lys-48 linkages, other forms of linkage for both mono- and poly-ubiquitin chains can occur, such as Lys6, Lys29/33 and Lys63, which regulate protein degradation as well as a wide array of other cellular activities in a proteolysis-independent manner. In addition, non-proteolytic Lys6 and Lys11 polyubiquitin linkages have been shown to be associated with neurodegenerative disorders (reviewed in (Bernassola, Karin et al. 2008)).

E6AP ubiquitin ligase

The E6AP ubiquitin ligase (also called UBE3A) is a member of the HECT E3 family. It is a 100 kDa protein that interacts with the HPV E6 protein. E6AP targets substrate proteins, including itself, for proteasomal degradation (de Bie and Ciechanover 2011). Autoregulation of E6AP via self-targeted degradation is cited as a mechanism for maintaining E6AP levels (Nuber, Schwarz et al. 1998, de Bie and Ciechanover 2011). Both loss and activation of E6AP functions are implicated in various human diseases, with Angelman Syndrome being the most well known (Nicholls and Knepper 2001, Beaudenon and Huibregtse 2008, Flashner, Russo et al. 2013). Abrogation of E6AP by deletion or mutation of the *UBE3A* gene locus within the 15q11–13 chromosome region is associated with Angelman syndrome, a neurological disorder (Kishino, Lalande et al. 1997, Sutcliffe, Jiang et al. 1997, Matentzoglou and Scheffner 2008).

Many of the naturally occurring mutations within the *UBE3A* gene introduce deletions that generate a truncated E6AP protein, lacking the intact HECT-domain. Although, approximately 10% of the genetic alterations correspond to point

mutations within the E6AP coding region (Jiang, Lev-Lehman et al. 1999), many of the point mutations represent loss-of-function alterations and are not able to ubiquitinate their substrate, however, in contrast to this, many retain the ability to form a thioester bond with ubiquitin (Cooper, Hudson et al. 2004). In addition, duplication of the *UBE3A* gene is thought to result in some cases of autism disorder (Schaaf, Sabo et al. 2011, Smith, Zhou et al. 2011), and recent studies have found that Protein kinase A (PKA)-mediated phosphorylation of E6AP at T485 residue inhibits the E6AP activity toward itself and other substrates. An autism-linked missense mutation disrupts this phosphorylation site resulting in enhanced ubiquitin activity and thus leading to synaptic dysfunction and autism pathogenesis (Yi, Berrios et al. 2015). Whether this phospho-regulation of E6AP has any relevance for the function of E6 remains to be determined.

Some of the E6AP protein's interactors include: RAD23A (Kumar, Talis et al. 1999), BLK (Oda, Kumar et al. 1999), MCM7 (Kuhne and Banks 1998), UBQLN1 and -2 (Kleijnen, Shih et al. 2000, Kleijnen, Alarcon et al. 2003), the estrogen receptor (Li, Li et al. 2006), TSC2 (Zheng, Ding et al. 2008), annexin A1 (Shimoji, Murakami et al. 2009), PML (Louria-Hayon, Alsheich-Bartok et al. 2009), peroxiredoxin 1 (Nasu, Murakami et al. 2010), Arc (Greer, Hanayama et al. 2010), and Ring1B (Zaaroor-Regev, de Bie et al. 2010). Amongst which Ring1B has been shown to be get ubiquitinated and degraded in proteasome dependent manner (Zaaroor-Regev, de Bie et al. 2010, Thatte and Banks 2017). However, there is still considerable debate about which of them are E6AP substrates and its precise mode of action.

E6AP, E6 and p53 association

The E6AP ubiquitin ligase was initially identified as an interacting partner of the E6 protein encoded by HPV-16 and HPV-18 (Huibregtse, Scheffner et al. 1991, Scheffner, Huibregtse et al. 1993, Beer-Romero, Glass et al. 1997). In the absence of E6, p53 degradation is mediated by the Mdm2 ubiquitin ligase (Haupt, Maya et al. 1997). However, in HPV-positive cells, E6 mediates degradation of p53 through its interaction with E6AP (Scheffner, Werness et al. 1990, Talis, Huibregtse et al. 1998). E6-E6AP interaction occurs through a leucine-rich motif (LXXLL) motif in the N-terminal region of E6AP (Chen, Hong et al. 1998, Elston, Napthine et al. 1998, Be, Hong et al. 2001). The structural and biophysical analyses of the E6 interaction with E6AP showed that the N-terminal E6 Zn^{2+} -binding domain primarily interacts with E6AP, whereas the C-terminal Zn^{2+} -binding domain interacts with p53, as shown in Figure 10 (Nomine, Charbonnier et al. 2003, Zanier, Charbonnier et al. 2005, Liu, Cherry et al. 2009, Zanier, Ruhlmann et al. 2010, Zanier, Charbonnier et al. 2013). The E6:E6AP complex binds to the DNA-binding domain of p53, which becomes rapidly ubiquitinated and is targeted to the 26S proteasome (Huibregtse, Scheffner et al. 1991). The potential to promote p53 degradation is a characteristic of E6 from the high-risk HPV types (e.g. HPV types 16 and 18). In contrast, the E6 proteins from low-risk HPV types do not stably interact with E6AP *in vitro*, however, *in vivo*, all alpha type HPV E6 proteins can associate with E6AP (Huibregtse, Scheffner et al. 1991). E6 proteins from all HPV genera except Alpha interact with MAML1 (which regulates notch signaling) over E6AP, suggesting that the differences in E6 interaction with MAML1 or E6AP is a major event in papillomavirus evolution (Brimer, Drews et al. 2017). Although several other targets of E6AP have been suggested as potential mediators of E6's tumorigenic activity (Liu, Yuan et al. 2005), the major contribution of E6AP to HPV-associated tumour development is thought to be achieved through the degradation of the p53 tumour suppressor.

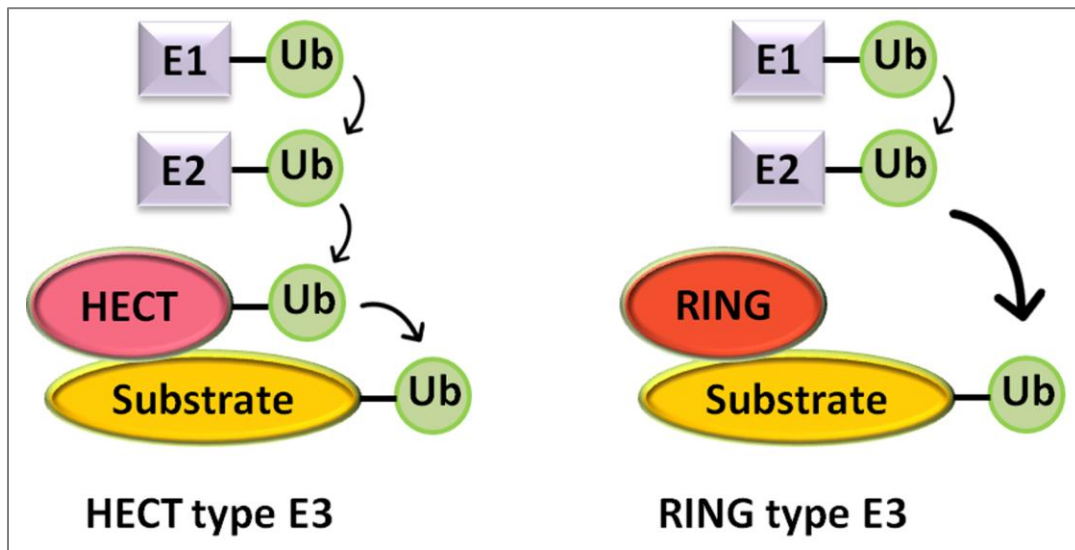


Figure 9. Different types of E3 ubiquitin-ligases.

The ubiquitin-ligase E3 is classified into two ubiquitin-ligase groups based on differences in domain structure, they are: HECT type and RING type. RING-type E3s add Ub directly from E2 to the substrate, functioning as a scaffold, whereas HECT type E3 itself binds with cysteine residue to form an intermediate that forms thioester bonds with ubiquitin.

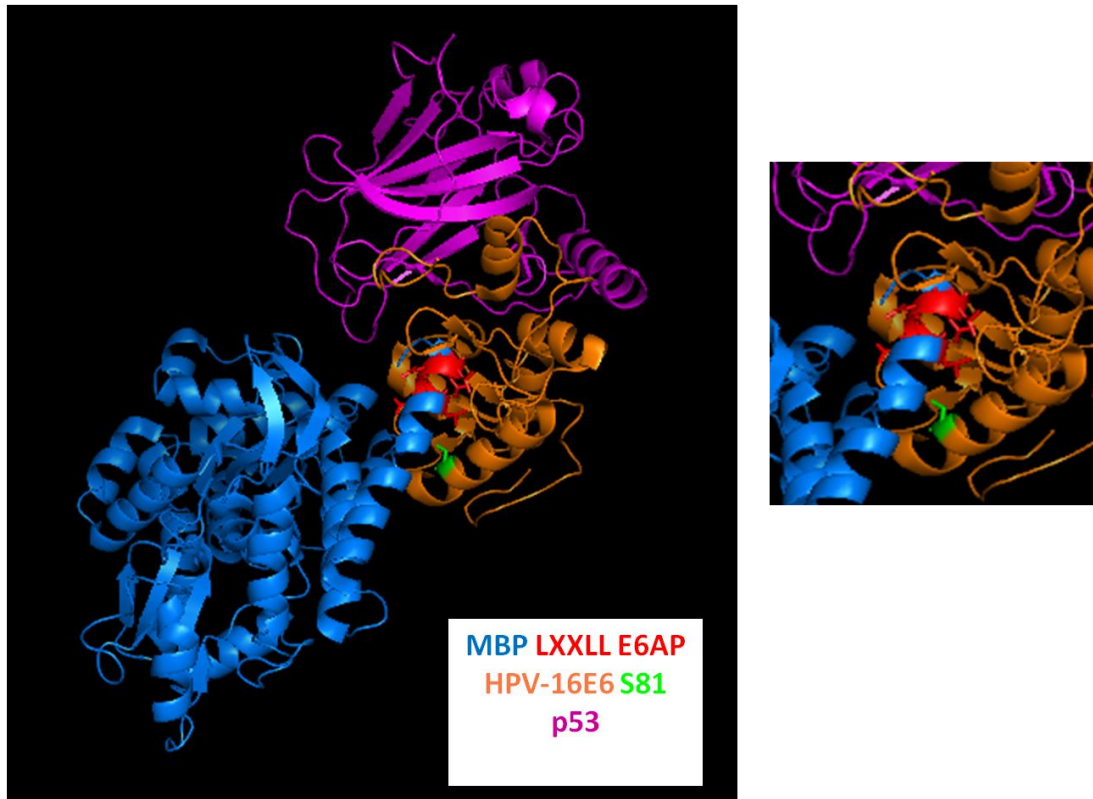


Figure 10. Ribbon diagrams representing the structures of E6/MBP-E6AP/p53.

A diagram showing E6/MBP-E6AP/p53 core structure observed in the asymmetric unit. Red: LXXLL peptide of E6AP fused to the C-terminus of MBP (Blue); HPV-16 E6 (Orange); HPV 16 E6 Ser81 residue (Green) and p53core (Pink) are shown.

DNA damage Response (DDR)

The DNA damage response (DDR) is a complex network of cellular signaling that is triggered by various endogenous or exogenous factors. The endogenous factors include the dysfunction of DDR repair or DNA replication proteins, and exogenous factors include ionizing radiation, UV light, exposure to DNA-damaging chemicals etc. During DNA replication, spontaneous DNA alterations can occur, which lead to the misincorporation of dNTPs, causing: deamination; insertion or deletion of DNA bases; DNA depurination, or modification of DNA bases by alkylation (illustrated in Figure 12) (Lindahl and Barnes 2000). Additionally, reactive oxygen species generated in cells also trigger the oxidation of nucleotides - and incorporation of oxidized nucleotides causes DNA damage (Shibutani, Takeshita et al. 1991, Kamath-Loeb, Hizi et al. 1997, Cooke, Evans et al. 2003, Hoeijmakers 2009, Jackson and Bartek 2009). Various forms of DNA damage can also be caused by the chemicals used in cancer therapy. These include cross-linking agents such as mitomycin C (Bizanek, McGuinness et al. 1992) and cisplatin (Brabec 2002), and topoisomerase inhibitors such as etoposide and camptothecin, which induce the formation of single-strand (SSB) and double-strand breaks (DSB) in the DNA (Hande 1998, Pommier 2006).

The DDR senses, signals and facilitates the repair of damaged DNA. The DDR pathways are activated in order to maintain genome stability through mechanisms that coordinate DNA repair, cell cycle progression, transcription, apoptosis, and senescence (Abraham 2001, Karagiannis and El-Osta 2004, Jackson and Bartek 2009, Brnzei and Foiani 2010, Ciccia and Elledge 2010, Marechal and Zou 2013). Any mutation in DNA repair genes can cause genomic instability and thus may lead to cancer. The two central pathways activated in response to DNA damage are the Ataxia Telangiectasia Mutated (ATM) pathway and the ATM and Rad-3 related

(ATR) pathway. The ATM and ATR proteins belong to the phosphatidylinositol 3-kinase-related kinase (PIKK) family of serine/threonine kinases. ATM is primarily activated in response to DSBs, whilst ATR is activated in response to a broad spectrum of DNA damage, including SSBs and a variety of DNA lesions that interfere with DNA replication.

ATM and ATR

The DSBs in the DNA lead to activation of the ATM pathway. The Mre11/Rad50/Nbs1 (MRN) complex binds to DNA at the site of the DSB and initiates the signals for ATM recruitment (Lee and Paull 2005). However, how exactly DSBs recruit activators of ATM remains unclear. This activation is through autophosphorylation of ATM at residue S1981, which results in the monomerization of ATM (ATM exist as dimers or oligomers in undamaged cells), and this has been shown to be essential for its activity (Bakkenist and Kastan 2003). Reactive oxygen species also trigger the activation of ATM, but in this case independently of the MRN complex (Guo, Kozlov et al. 2010). ATM further signals to various downstream substrates in order to initiate the DNA repair signaling. The ATM kinase recognizes 'S/T-Q' motifs on its substrates (Kim, Lim et al. 1999); one very well characterized substrate of ATM is Chk2, which is phosphorylated by ATM at the Thr68 residue in response to DSBs (Chaturvedi, Eng et al. 1999, Matsuoka, Rotman et al. 2000). In addition to this, ATM also phosphorylates and activates several other signaling proteins such as p53, BRCA1, FANCD2, H2AX and Nbs1 (Shiloh and Ziv 2013). ATM-responsive pathways are involved in activation of various downstream signaling networks: p53 activation, NF- κ B and microRNA activation, all of which are essential for gene-transcription regulation in response to DNA damage (Turenne, Paul et al. 2001, Wu, Shi et al. 2006, Zhang, Wan et al. 2011). ATM activation has also been shown to be involved in nucleosome

remodeling and chromatin relaxation (Ziv, Bielopolski et al. 2006, Goodarzi, Noon et al. 2008, Polo, Kaidi et al. 2010, Goodarzi, Kurka et al. 2011, Moyal, Lerenthal et al. 2011)

ATR activation is stimulated in response to SSBs in the DNA. Unlike ATM, the ATR-ATRIP heterodimer does not interact with DNA directly; instead this interaction occurs with the help of replication protein A (RPA), promoting ATR localization to the sites of replication stress (Zou and Elledge 2003). The activity of ATR is important to initiate several signaling cascades in response to replication stress. The autophosphorylation of ATR at Thr1989 is dependent on the formation of the RPA-ATRIP complex (Liu, Shiotani et al. 2011). Activation of ATR is a multistep process, and where the ATR-ATRIP recognizes the SSB-bound RPA, the 9-1-1 (Rad9, Hus1, and Rad1) complex is simultaneously loaded at the junctions of ssDNA. This promotes TopBP1-mediated ATR activation, which is required for the stabilization of replication forks (Delacroix, Wagner et al. 2007, Cotta-Ramusino, McDonald et al. 2011). The well-characterized downstream effector of ATR is Chk1, which is activated upon ATR-mediated phosphorylation on residues S317 and S345 (Liu, Guntuku et al. 2000, Zhao and Piwnica-Worms 2001). Activation of Chk1 has been shown to be critical for intra-S and G2/M checkpoint responses. These events are illustrated in Figure 11.

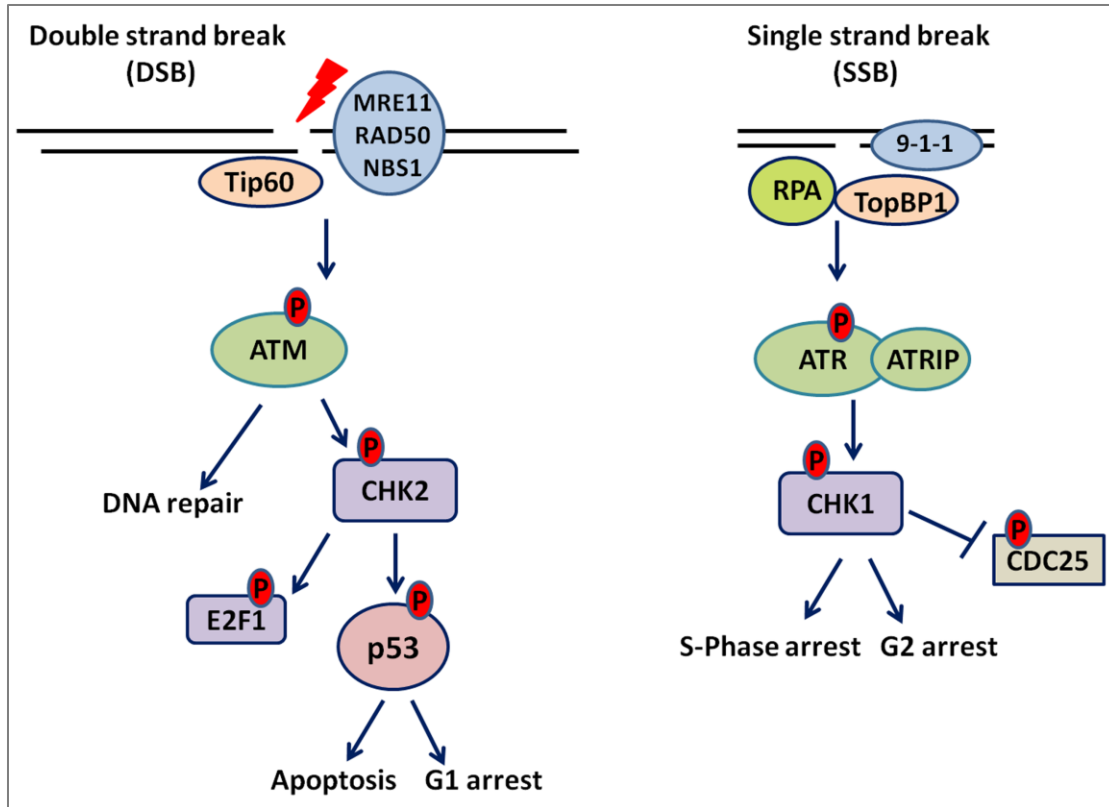


Figure 11. DNA damage response (DDR) pathway.

DNA damage response is a signaling cascade that is coordinated by various proteins and they are categorized as sensors, transducers and effectors. Double strand break (DSB) can be sensed by the MRN complex (sensor) to recruit and activate transducer ATM to activate CHK2 (effector) with the help of DDR mediators such as Tip60. The single strand break (SSB) can be detected by sensor protein, RPA and 9-1-1 complex, to recruit transducer ATR, to activate CHK1 (effector), with the help of mediators TopBP1. The p53 and CDKs are the downstream substrates in response to DSB and SSB respectively.

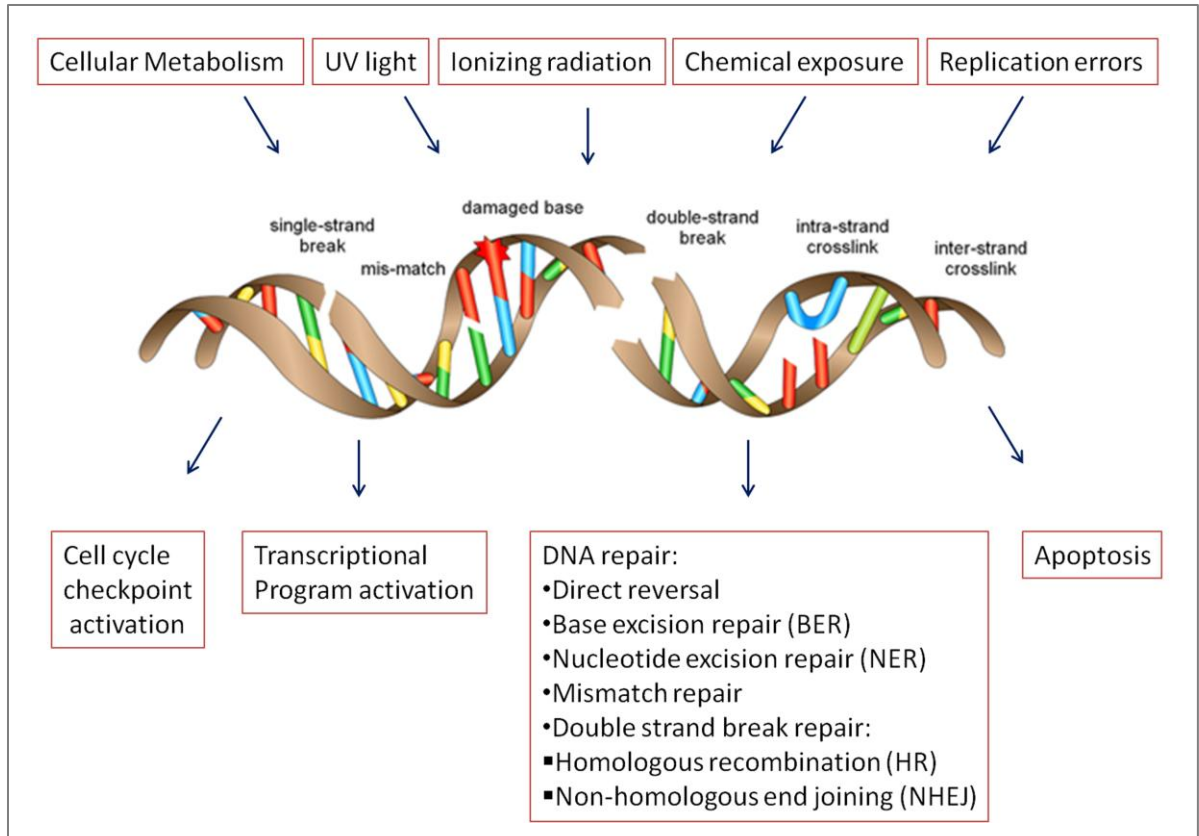


Figure 12. Different intrinsic as well as extrinsic factors involved in causing DNA damage.

DNA damage includes single strand break, base miss-match, double strand break, AP-site intra-strand crosslink and inter-strand crosslink. Based on the type of DNA damage, different downstream pathways are activated including cell cycle check point and transcriptional program activation, DNA repair or apoptosis.

Oxidative stress and DNA damage response

The imbalance between the production of reactive oxygen species (ROS) and antioxidants is termed oxidative stress. The oxygen molecule (O_2), the superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2) are all classified as ROS (Ames, Shigenaga et al. 1993, Agnez-Lima, Melo et al. 2012). Normal cellular processes also generate ROS during oxidative phosphorylation in the mitochondria and during the oxidation of long-chain fatty acids in peroxisomes. (Berquist and Wilson 2012). Hypoxia is another contributing factor for induction of DDR thereby inducing rapid replication arrest (Pires, Bencokova et al. 2010). Exogenous agents such as ionizing radiation (IR), ultraviolet (UV) radiation, or chemotherapeutic agents can also trigger ROS production in cells. (Riley 1994, Cook, Gius et al. 2004, Berquist and Wilson 2012, Dizdaroglu 2012). As a consequence, cells have evolved an elaborate defense system against ROS in the form of antioxidants such as glutathione, vitamin C and vitamin E etc. (Masella, Di Benedetto et al. 2005).

Various different types of DNA damage or replication stress are induced upon oxidative stress, including sugar moiety damage, Apurinic/apyrimidinic (AP) sites, purine and pyrimidine base damage, SSBs, DSBs, DNA intrastrand/interstrand crosslinks, protein–DNA crosslinks, nucleotide mismatched bases, stalled DNA replication forks, and oxidatively-generated clustered DNA lesions (OCDLs) (Ames and Saul 1986, Ames 1989, Lindahl 1993, Hoeijmakers 2009, Ciccia and Elledge 2010). In response to oxidative stress-induced DNA damage, a variety of DNA repair and DNA damage response (DDR) pathways are employed by cells, and these are critically essential for the maintenance of genome integrity (Bartek and Lukas 2003, Friedberg 2003, Barzilai and Yamamoto 2004, Ciccia and Elledge 2010, Chen, Li et al. 2012) (Figure 12). These pathways include: base excision repair (BER)/single-strand break repair (SSBR), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous

end joining (NHEJ) (Bartek and Lukas 2003, Friedberg 2003, Berquist and Wilson 2012). The ATM-Chk2 and ATR-Chk1 pathways are also activated in response to oxidative stress-induced DNA damage (Cimprich and Cortez 2008, Chen, Li et al. 2012).

DNA damage signaling and cell-cycle checkpoints

The cell cycle is a series of highly ordered processes in which cell cycle-checkpoint proteins play critical roles (Elledge 1996). The Cyclin-dependent kinases (CDKs), their cyclin partners, protein kinases, and phosphatases govern the progression of the cell cycle to the next phase (Nurse 2000). Activation of cell cycle checkpoint kinases, Chk1 and Chk2, by ATM and ATR respectively, suppresses the activity of the CDK1/cyclin B complex and thus the cell's mitotic entry is inhibited (Nigg 2001, Smits and Medema 2001, Melo and Toczyski 2002). Chk1 activation by ATR kinase in response to DNA damage leads to cell cycle arrest in S phase, which is achieved by Chk1 phosphorylation of Cdc25A and its consequent degradation, resulting in the inhibition of CDK2 (Sorensen, Syljuasen et al. 2003, Carrassa, Sanchez et al. 2009). Knockdown and inhibition of Chk1 is associated with increased SSBs, aberrant fork structures, accumulation of DSBs, and increased phosphorylation of ATR targets (Syljuasen, Sorensen et al. 2005, Petermann, Woodcock et al. 2010). On the other hand, Chk2 functions as an effector by phosphorylating additional key substrates, such as the transcription factor p53 (Chehab, Malikzay et al. 2000, Shieh, Ahn et al. 2000), the cell-cycle regulating phosphatases Cdc25A (Falck, Mailand et al. 2001) and Cdc25C (Peng, Graves et al. 1997), Mdm2 (Shieh, Ahn et al. 2000) and BRCA1 (Lee, Collins et al. 2000). In addition, the Chk2 phosphorylation of transcription factor E2F1 on serine 364 has been shown to be involved in transcriptional upregulation of the proapoptotic proteins Apaf-1 and p73 (Stevens, Smith et al. 2003).

Activation of DDR by viruses

Studies have shown that ATM signaling is essential for efficient lytic viral DNA replication of various human herpesviruses, including HSV (Lilley, Carson et al. 2005), CMV (E, Pickering et al. 2011), EBV (Li, Zhu et al. 2011, Hagemeyer, Barlow et al. 2012), and MHV68 (Tarakanova, Leung-Pineda et al. 2007, Tarakanova, Stanitsa et al. 2010). The replication of polyomaviruses is also dependent on ATM kinase activity (Shi, Dodson et al. 2005). Adeno-associated virus (AAV) have been shown to activate the DNA-PK pathway during coinfection with adenovirus as a helper (Cervelli, Palacios et al. 2008, Schwartz, Carson et al. 2009), and this activation is important for its replication (Choi, Nash et al. 2010). A number of reports show a role of ATM activation by HIV-1 during infection (Daniel, Marusich et al. 2005, Lau, Swinbank et al. 2005). Activation of DNA-PK pathway also potentially plays an important role in retroviral integration (Daniel, Katz et al. 1999). This suggests that many diverse viruses use DDR signaling for their replication. Figure 13 illustrates different viral proteins involved in modulating the DDR pathway.

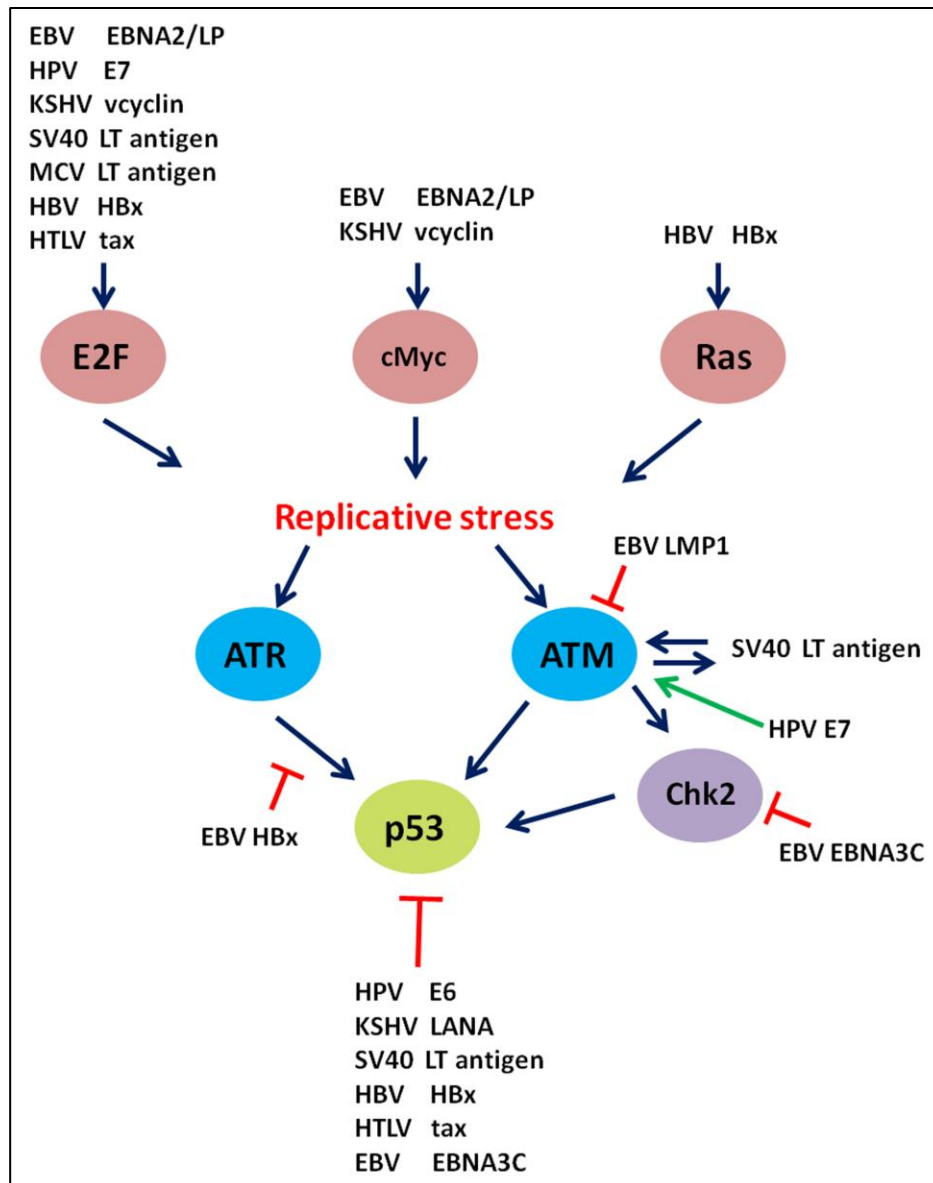


Figure 13. Crosstalk between viral oncoproteins and the host DDR.

Viral oncoproteins activate E2F, cMyc and Ras cellular oncogenes in order to enter or re-enter the cell cycle, thereby inducing replicative stress and DNA damage. In response to DNA damage, ATM and ATR kinases are activated which regulate downstream signaling including activation of Chk2 and p53. Tumour virus oncoproteins modulate the function of DDR components by activating or suppressing their expression or activity. Different viral proteins modulating the components of DDR are shown.

HPV and the DNA Damage response

Cells containing the high-risk HPVs exhibit constitutive activation of the ATM pathway in the absence of external DNA damage. Studies in HPV-31, HPV-16 and HPV-18-containing differentiating HFK cells showed activated ATM and its downstream substrates such as Chk2, Nbs1 and BRCA1 (Moody and Laimins 2009). Activation of the ATM-DDR response pathway was also shown to be required for amplification of HPV-31 genomes in differentiated cells (Moody and Laimins 2009), and different viral proteins such as E1 and E7 have also been shown to be essential for the recruitment of DSB repair factors to HPV replication centres (Fradet-Turcotte, Bergeron-Labrecque et al. 2011, Sakakibara, Mitra et al. 2011, Gillespie, Mehta et al. 2012, Reinson, Toots et al. 2013).

DDR activation during HPV maintenance, replication and amplification

HPV genome replication is dependent on the host cell machinery and the virus uses the components of the DDR for limited amplification of the viral genome in the basal layer. As noted, the HPV viral DNA replication is dependent on the E1 and E2 proteins, and their expression is required for the formation of replication foci that recruit components of the DDR (Fradet-Turcotte, Bergeron-Labrecque et al. 2011, Sakakibara, Mitra et al. 2011, Reinson, Toots et al. 2013). Recent studies have shown that the E2 protein binds to host chromatin in complex with BRD4 at the fragile sites in the genome (sites of replication stress) (Jang, Shen et al. 2014). This interaction is essential for viral genome maintenance and nucleation of replication foci in vegetative viral amplification; however, it varies amongst different HPV types.

HPV genomes are replicated in S-phase, in synchrony with host cell DNA replication, for viral genome maintenance. In this phase, both ATM and ATR signaling is activated, although inhibition of ATM does not affect the viral genome

maintenance, but it is critical for genome amplification only in differentiated cells, which occurs in G2/M like phase (Moody and Laimins 2009). However, this activation is critical for the high-risk HPV- life cycle. Recent studies have shown that ATM and downstream homologous recombination components associate in discrete nuclear foci along with the replicating viral genomes, in both undifferentiated and differentiated cells (Gillespie, Mehta et al. 2012, McKinney, Hussmann et al. 2015).

A number of studies in W12 cells containing episomal copies of HPV-16 genome demonstrated that siRNA-mediated reduction of ATM, ATR, Chk1 and several other DDR components results in a 40%–50% reduction in HPV-16 copy number, suggesting that the ATM-ATR pathway is the canonical pathway activated by HPV for viral genome maintenance and amplification (Edwards, Helmus et al. 2013, Edwards, Vidmar et al. 2013).

ATM activation

Normally, the ATM pathway is activated by Tip60-mediated acetylation. Studies have shown that the Tip60 levels are increased in the cells containing the whole viral genome, where various viral gene products are expressed, although E6 has been reported to degrade Tip60 (Jha, Vande Pol et al. 2010, Hong, Dutta et al. 2015). Also, in HPV-positive cells, expression of Tip60 is required for ATM activation as well as for genome amplification upon differentiation (Hong, Dutta et al. 2015). Studies in HPV-16 and HPV-31 have demonstrated that expression of E7 is sufficient to induce the recruitment of the MRN components to the site of DNA damage, which further facilitates the activation of ATM by autophosphorylation (Anacker, Gautam et al. 2014). ATM activation signals to its downstream effectors that are involved in DNA repair pathways. In addition to this, ATM also phosphorylates a cohesin protein, SMC1, in response to DNA damage. Its expression is elevated in HPV-positive cells and this also has been shown to be required for

genome amplification. The phosphorylated SMC1 is localized to distinct nuclear foci containing γ -H2AX and pCHK2 and it binds directly to viral DNA at conserved CTCF sites (Mehta, Gunasekharan et al. 2015). Taken together, these findings suggest that there is a critical requirement for ATM activation during the viral life cycle; however, it still remains unclear how exactly the ATM pathway promotes the amplification of viral genomes in differentiated cells.

ATR activation

In addition to activating the ATM pathway, HPV also activates the ATR pathway. Recent studies in HPV-18 have shown that the transient replication of viral genomes triggers activation of an ATR-dependent DNA damage response that subsequently activates ATR pathway components such as ATRIP and TopBP1 at viral replication centers (Reinson, Toots et al. 2013). This ATR activation is a result of replication stress caused by the initial amplification of viral genomes during the establishment-phase. In addition, ATR activation is linked to the stable maintenance of viral episomes in primary keratinocytes. The studies in primary keratinocytes showed that both the total and phosphorylated levels of ATR and its substrate Chk1, are increased in the cells containing high-risk HPV genomes, as well as in cells expressing E6 and E7 alone (Yang, Liu et al. 2016). Whole genome studies showed that the transcription of the Topoisomerase II β -Binding Protein 1 (TopBP1) gene is regulated by STAT-5 to activate the ATR Pathway and promote HPV replication. The inhibition of TopBP1 results in loss of ATM/Chk1 activation, leading to a reduction in the stable maintenance of viral genomes in undifferentiated cells (Hong, Cheng et al. 2015). Recent studies showed that pharmacological inhibition of either pATR or pChk1 by small molecule inhibitors leads to loss of genome amplification in differentiating cells (Edwards, Helmus et al. 2013, Hong, Cheng et al. 2015).

Taken together, these findings highlight the importance of both ATM and ATR for differentiation-dependent amplification and replication of HPV genomes. It is

possible that HPV activates ATR pathways to maintain replication fork integrity during the stress of viral replication, to activate stalled replication forks for continued replication of viral genomes and also to recruit replication factors at viral replication centers.

E6/E7 and DDR

The E6 and E7 are smart players of the HPV life cycle as they both activate the DDR pathways, at the same time as they inhibit the downstream consequences. E7 plays a critical role in promoting DDR activation for viral replication, whilst E6 targets and inhibits the subsequent effectors of DDR, such as the p53 tumor suppressor, thus avoiding growth arrest or apoptosis. E7 also elevates the levels of homologous recombination proteins, including BRCA1 and RAD51 throughout the viral life cycle, together with NBS1. HPV-31 genome studies showed that these events are required for the amplification of viral genomes in differentiated cells (Anacker, Gautam et al. 2014, Chappell, Gautam et al. 2015). In addition, SIRT1 deacetylase regulates the recruitment of both NBS1 and RAD51 to viral genomes, thus controlling homologous recombination in productive viral replication (Langsfeld, Bodily et al. 2015). E7 deregulates the E2F1 transcription factor by targeting pRB, and this induces the expression of Chk2, promoting viral replication (Rogoff, Pickering et al. 2004). Moreover, studies in HPV 16 showed that E7 induces the degradation of claspin, which is a Chk1 binding protein, thus promoting mitotic entry in the presence of an activated DDR (Spardy, Covella et al. 2009).

Normally, activation of DDR pathways signal to p53 for concomitant growth arrest, however, the ability of E6 to degrade p53 is thought to be crucial for genome maintenance (Thomas, Hubert et al. 1999, Flores, Allen-Hoffmann et al. 2000, Park and Androphy 2002). Furthermore, inactivation of p53, or expression of a dominant-negative form of p53, rescues this effect (Lorenz, Rivera Cardona et al. 2013, Brimer and Vande Pol 2014). HPV modulated DDR pathway is illustrated in Figure 14.

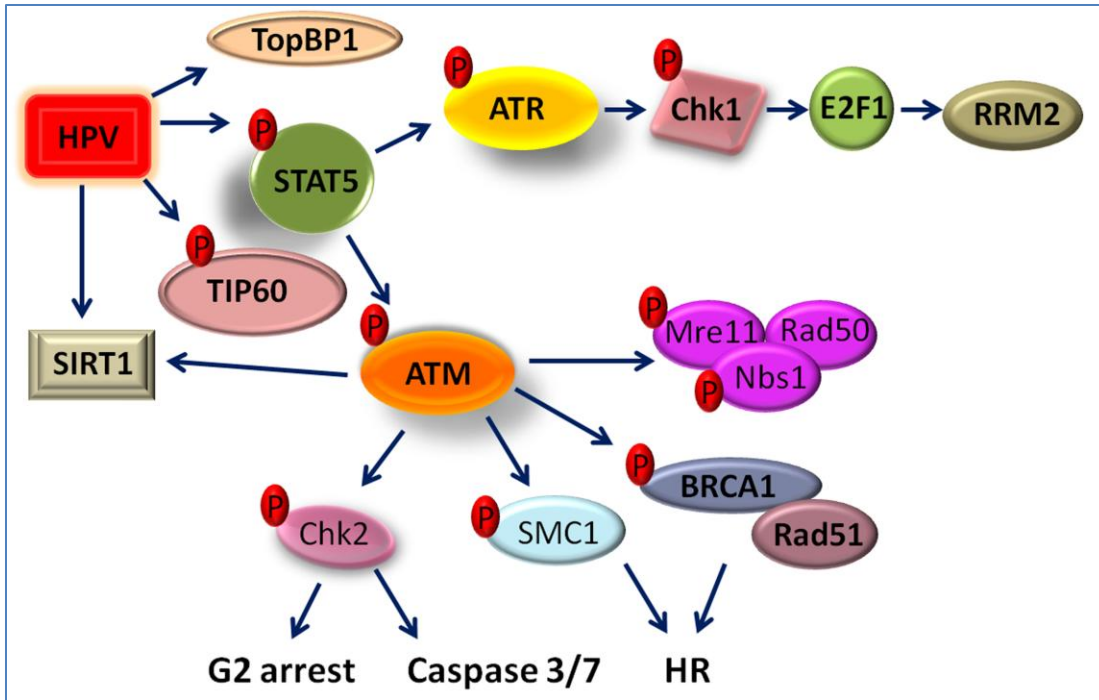


Figure 14. HPV mediated modulation of the ATM and ATR DNA damage response pathways. HPV-induced activation of ATM is mediated via immune regulator STAT5 as well as TIP60. ATM activation further stimulates downstream effectors such as the Chk2, and HPV may utilize this activity to promote G2 arrest upon differentiation and can also use it for Caspase 3/7 activation. ATM also signals to MRN complex, Rad51, BRCA1 and SMC1 which are involved in regulation of homologous recombination. ATR activation in HPV positive cells is triggered by E7-induced replication stress and requires a STAT5-directed increase in TopBP1. ATR/Chk1 activation further increases E2F1, which drives expression of RRM2, resulting in increased dNTP pools to facilitate productive viral replication.

AIMS OF THIS THESIS

Previous studies have shown how E6 phosphorylation can alter substrate selection but there was no information on the biological consequences, or on when this might occur in vivo. Furthermore, there was no information on whether phosphorylation of E6AP might also be relevant for E6 activity. My aims were:

- To determine when E6 was phosphorylated in vivo.
- To identify which kinases were responsible.
- To identify the sequence constraints controlling kinase and 14-3-3 recognition of E6.
- To investigate the biological consequences of E6 phosphorylation.
- To determine how phosphorylation of E6AP could likewise affect E6 function.
- To begin to investigate differences in how different cancer-causing E6 oncoproteins might respond to these different signaling pathways.

CHAPTER 2: MATERIALS AND METHODS

Cell culture and transfection

HeLa, H1299, HEK293 wild type (ATCC) and E6AP/KO HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and glutamine (300 µg/ml). All these cell lines were cultured in an incubator at 37°C with 10% CO₂.

The NIKS (Normal Immortalised Keratinocytes) (Allen-Hoffmann, Schlosser et al. 2000), NIKS 16 E6 and NIKS 16 E6ΔPBM cells (Nicolaidis, Davy et al. 2011) were maintained in medium composed of 3 parts Ham's F12 medium to 1 part DMEM, and supplemented with: 5% fetal bovine serum (FBS), adenine (24 µg/ml), cholera toxin (8.4 ng/ml), epidermal growth factor (10 ng/ml), hydrocortisone (2.4 µg/ml), insulin (5 µg/ml), penicillin-streptomycin (100 U/ml), and glutamine (300 µg/ml). NIKS cell lines were cultured in an incubator at 37°C with 5% CO₂.

HEK293 and H1299 cells were transfected using the calcium phosphate precipitation method (Wigler, Pellicer et al. 1979). Cells were harvested after 18h.

HeLa cells were transfected with siRNA against the appropriate genes using Lipofectamine RNAiMax transfection reagent (Invitrogen). The samples were analyzed 72h post-transfection.

Plasmids and cloning

The pCDNA-18E6 R153G and pGWI-HA:18E6 ΔPBM were provided by Boon SS. The pcDNA3 FLAG- p53 (Pim, Massimi et al. 1997), pGWI:HA-Dlg (Gardioli, Galizzi et al. 2002), pGWI HA-18E6 and pGWI HA-16E6 were kindly provided by Ron Javier. Flag-PML plasmids have been described previously (Guccione E. et al. 2004).

HA-tagged HPV-31 E6 was sub-cloned into the pGWI vector within compatible HindIII and EcoRI restriction enzyme sites and pcDNA3:HA-Ub(n) has been described previously (Tomaic, Pim et al. 2011).

The plasmids used for luciferase reporter assays are as follows: p21-Luciferase, BAX-Luciferase, MDM2-Luciferase, PUMA-luciferase and pCMV-Renilla luciferase were kind gifts from Dr. Giannino Del Sal, and are described in REFS.

The pCS2-6Myc-Ring1B (Zaaroor-Regev, de Bie et al. 2010) was a kind gift from Prof. Aaron Ciechanover. The Ring1B was sub-cloned into the pGWI vector using compatible HindIII and EcoRI restriction enzyme sites. The 14-3-3 inhibitor plasmid, Myc-Difopein, was a kind gift from Haian Fu (Masters and Fu 2001). The Myc-E6AP, Myc-E6AP T485A, Myc-E6AP T485E were kind gifts from Mark Zylka (Yi, Berrios et al. 2015).

The plasmids expressing GST, GST-HPV-18E6, GST-HPV-18E6R153A and GST-HPV-18E6T156E fusion proteins (Cloned in pGEX2T vector) have been described previously (Boon and Banks 2013, Boon, Tomaic et al. 2015). The PBM mutants were generated using the GeneArt Site-Directed mutagenesis kit (Invitrogen).

The pGEX2T-E6AP expression plasmid has been described previously (Tomaic, Pim et al. 2011). The pGEX2T-E6APT485A mutant was generated using the GeneArt Site-Directed mutagenesis kit (Invitrogen).

The HPV-31 E6 GST-fusion proteins have been described previously (Boon and Banks 2013, Boon, Tomaic et al. 2015). HPV-31 E6 deletion and substitution mutants were generated in the pGEX2T construct using the GeneArt Site-Directed mutagenesis kit (Invitrogen).

The specific mutation or the substitution and the primer sequences are shown in the table below.

E6 Type	Mutation	Primer sequences
18	ET*	F: CGACGCAGAGAAACATGATATTAAGTATGCATGG
		R: CCATGCATACTTAATATCATGTTTCTCTGCGTCG
	ETQ*	F: CGCAGAGAGAAACACAATGATATTAAGTATGCATGG
		R: CCATGCATACTTAATATCATTGTGTTTCTCTGCG
	ETAA	F:CGACGCAGAGAAACGCAGCATAATATTAAGTATGC
		R:GCATACTTAATATTATGCTGCTGTTTCTCTGCGTCG
	S82D	F: GAGAATTAAGACATTATGACGACTCTGTGTATGG
		R: CCATACACAGAGTCGTCATAATGTCTTAATTCTC
	Δ PBM	F: CCAACGACGCAGATAAACACAAGTATAA
		R: TTATACTTGTGTTTATCTGCGTCGTTGG
16	Δ PBM	F: CAAGAACACGTAGATAAACCCAGCTG
		R: CAGCTGGGTTTATCTACGTGTTCTTG
31	S82G	F: GATGGTATAGATATGGCGTGTATGGAACAAC
		R: GTTGTTCCATACACGCCATATCTATACCATC
	S82A	F: AGATGGTATAGATATGCTGTGTATGGAACAACA
		R: TGTGTGTTCCATACACAGCATATCTATACCATCT
	S82D	F: AGATGGTATAGATATGATGTGTATGGAACAACA
		R: TGTGTGTTCCATACACATCATATCTATACCATCT
	Δ PBM	F: GGAGAAGACCTCGTACTTAAACCCAAGTGTAAG
		R: CTTACACTTGGGTTTAAGTACGAGGTCTTCTCC
E6AP	T485A	F: GTGAACGAAGAATCGCTGTTCTCTACAGC
		R: GCTGTAGAGAACAGCGATTCTTCGTTAC

gRNA design and CRISPR/Cas9 mediated gene targeting

Two different gRNAs were designed against the E6AP gene using the DNA2.0 software. The gRNA primer sequences containing BbsI enzyme sticky ends are as follows:

gRNA1 : 5' CACCGGTTTCCAGGGGGTCCACTCG 3'
5' AAACCGAGTGGACCCCCTGGAAACC 3'

gRNA2: 5' CACCGAAGTGGTTTTTCGACAATCCA 3'
5' AAACCTGGATTGTCGAAAACCACTTC 3'

gRNA1 binds at genomic locus 15:25370913-25370932 and gRNA2 at 15:25370857-25370876. The gRNAs were cloned into pSpCas9(BB)-2A-Puro (PX459) using the BbsI restriction enzyme site. The Cas9-puro plasmids containing gRNA were then transfected into HEK293 cells and single-cell clones were selected using 10µg/ml puromycin (Sigma) for 4-5 weeks. The mutation in E6AP was verified by picking individual clones, and analyzing the relevant region of the E6AP genomic DNA by PCR and DNA sequencing. Verification of the loss of E6AP was performed by western blotting for E6AP.

Inhibitors, chemicals and reagents

The inhibitors and chemicals used to treat HeLa and C4-1 cells: 5µM AZ-20 ATR inhibitor (for 15h), 5µM KU-60019 ATM inhibitor (for 15h), 100nM UCN01 Chk1 inhibitor (for 15h), 5 µM Chk2 inhibitor (for 15h), 500µM hydrogen peroxide (H₂O₂) solution (for 5h), 5mM N-acetyl-cysteine (NAC) (for 15h), 100µg/ml Cycloheximide solution (for 4h), 200ng/ml Nocodazole (for 15h), 2.5mM Thymidine (for 15h), 10 µM H-89 PKA inhibitor (for 15h), 20nM MG-132 (for 15h), (All from Sigma Aldrich).

The chemotherapeutic reagents used were: 5µM Teniposide (for 15h), 50-100µM Cisplatin as indicated (for 15h), 10µM Triapine/3-AP (for 15h), 10µM Etoposide (for 15h) (All from Sigma Aldrich). The concentration of all above chemicals was

optimized based on the cell toxicity and their efficacy for each cell line (By also considering the available information from literature).

Antibodies

Following primary antibodies were used: Anti-Myc mouse monoclonal antibody (Santa Cruz Biotechnology); anti-FLAG mouse monoclonal antibody, clone M2 (Sigma); anti- β -galactosidase mouse monoclonal antibody (Promega); anti-HA mouse monoclonal antibody, clone HA-7, conjugated to Peroxidase (Sigma-Aldrich); anti-14-3-3 γ and 14-3-3 ζ rabbit antibodies (Santa Cruz Biotechnology); anti- α -actinin rabbit antibody (Santa Cruz); mouse monoclonal anti-p53 (DO-1) (Santa Cruz); rabbit polyclonal anti-pChk2(Thr68) (Novus Biologicals); rabbit polyclonal anti-pChk1(S317) and mouse monoclonal anti-Chk1 (Abcam), the rabbit polyclonal HPV-18 E6 phospho-specific antibody (custom-made by Eurogentec) was generated using H2N-RQERLQRRRET(PO3H2)QV-COOH peptide in rabbits and subjected to affinity purification. The mouse anti-HPV-18 E6 (Arbor Vita) have been described previously (Boon and Banks 2013, Boon, Tomaic et al. 2015). The appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Dako) were used

For immunofluorescence, mouse monoclonal anti-E6AP (BD Transduction labs), rabbit polyclonal anti- p53 (Santa Cruz Biotechnology) and chicken anti-(pT485) E6AP (A kind gift from Mark Zylka) were used. Appropriate Alexa-Fluor secondary antibodies (Life technologies) were used.

Production and purification of GST-fusion proteins

The appropriate expression plasmids were transformed into E. coli strain DH5- α . The clones harboring plasmids were grown in 40ml of Luria Broth (LB) culture media containing 75 μ g/ml Ampicillin (Sigma) overnight at 37°C. The overnight grown cultures were then transferred into 400ml of LB culture media containing 75 μ g/ml Ampicillin and incubated at 37°C for 1h. The isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma) to a final concentration of 1 nM was added to

induce recombinant protein expression and the culture was incubated for approximately 3h at 37°C on shaker. Post IPTG treatment, the bacteria were harvested by centrifugation at 5000 rpm for 5 minutes. Supernatant was discarded and the bacterial pellets were lysed with 5-10ml of 1X PBS containing 1% Triton X-100, and sonicated once/twice for 30 seconds at 80% amplitude. The lysates were then centrifuged again at 10000 rpm for 15 minutes. The supernatants were collected and incubated with glutathione-conjugated agarose beads on a rotating wheel overnight at 4°C. The GST-fusion protein containing beads were then centrifuged at 2000 rpm for 1 minute and the supernatant was discarded. The beads were washed thrice with 1X PBS containing 1% Triton X-100. The GST-fusion protein containing beads were then stored with 20% glycerol at -20°C.

Luciferase assay

The H1299 cells were transfected with appropriate plasmids expressing the luciferase reporters, and cell lysates were collected 24h post-transfection in the lysis buffer provided by the manufacturer. The luciferase assay was performed using the Dual-Luciferase reporter assay system (Promega), according to the manufacturer's instructions. The Firefly luciferase and Renilla luciferase readings were taken using a TD20/20 Luminometer (Turner Designs).

***In vitro* binding assays**

In vitro phosphorylation of the GST-fusion proteins was carried out in the presence of 10µM non-radiolabelled ATP (NEB). After extensive washes, both the phosphorylated and non-phosphorylated GST-fusion proteins were incubated with 100ng of purified Human recombinant 14-3-3γ protein (Abcam) at 4°C for 1h. The samples were then washed and analysed by SDS-PAGE and western blotting.

***In vitro* translation**

Proteins were translated *in vitro* using a TNT kit (Promega) and radiolabeled with [35S]methionine (Perkin Elmer). The purified GST-fusion proteins were then

incubated with *in vitro*-translated proteins for 1hr at 4°C. Proteins were washed with 1X PBS containing 0.1% NP-40, and were analyzed by SDS-PAGE and autoradiography.

***In vitro* phosphorylation assays**

The GST-fusion proteins were washed with kinase buffer (25 mM Tris HCl pH 7.5, 10mM MgCl₂) containing 0.1% NP-40), prior to performing the phosphorylation reactions.

Chk1 and Chk2 kinase assay: The Chk1 and Chk2 kinase assay was performed using the Chk1 and Chk2 kinase assay system (Promega). The proteins were then incubated with the respective full-length kinases (0.1-0.2µg) diluted in 1X reaction buffer A (provided by the manufacturer), together with 2.5µCi [32p]-γ-ATP for 1hr at room temperature.

PKA kinase assay: *in vitro* phosphorylation of the GST-fusion proteins was carried out using 20µl kinase buffer containing 2.5µCi [32p]-γ-ATP and 25 Units of cAMP-Dependent Protein Kinase Catalytic Subunit (Promega) for 30mins at 30°C. The buffer composition used for phosphorylation assay: 25 mM Tris HCl, pH 7.5, 10mM MgCl₂ and 70mM NaCl.

AKT kinase assay: *in vitro* phosphorylation of the GST-fusion proteins was carried out using 0.2µg of full-length AKT1 diluted in 1X Reaction Buffer A from the AKT1 Kinase enzyme system (Promega) for 45 minutes. The buffer composition used for phosphorylation assay: 25 mM Tris HCl, pH 7.5, 10mM MgCl₂ and 5mM DTT.

The samples were then washed with kinase buffer containing 0.1% NP-40 and analysed by SDS-PAGE and autoradiography.

Ubiquitination assays

The relevant plasmids were transfected into E6AP-null HEK293 cells and after 24h cell lysates were prepared, followed by immunoprecipitation using anti-HA- or anti-

FLAG-conjugated agarose beads to pull down ubiquitin-conjugated proteins (Sigma-Aldrich), as described previously (Tomaic, Pim et al. 2009). The beads were then washed and polyubiquitinated HHR23A or Ring1B protein, respectively, was detected using western blotting.

Half-life experiments

After 24hrs of transfection, the cells were treated with cycloheximide (50µg/ml in dimethyl sulfoxide [DMSO]) to block protein synthesis at different time points. DMSO alone-treated cells were used as the control. Cells were then lysed with SDS-sample buffer and samples were analysed by western blotting.

Cell synchronization and FACS analysis

The HeLa cells were treated with 2.5mM thymidine for 15hrs and washed thoroughly with 1X PBS and supplemented with complete media. After 9hrs the second dose of thymidine was added to block the cells in the G1/S phase. The cells were washed with PBS after 15hrs and the medium was replaced. The cells were then harvested at different time points as follows: 0h (G1/S phase), 3hrs (S phase), 8hrs (M1 phase), 9/10hrs (M2 phase) and 12hrs (Next G1 phase). The cell cycle phase analysis was done using propidium iodide staining and FACS analysis, using a FACScalibur Cell Sorter (Becton Dickinson).

Immunofluorescence assay

HeLa cells seeded on glass coverslips were transfected with siRNAs against appropriate genes for 72h. The cells were then washed with 1X PBS and fixed using 4% paraformaldehyde for 15mins and permeabilized using PBS containing 0.1% Triton X-100 for 5mins. The cells were briefly incubated with 100mM glycine. Immunostaining was performed by incubating the coverslips in PBS containing appropriate antibodies for 2h at 37° C in a humidified chamber. The coverslips were then washed thrice with PBS and incubated with respective fluorophore conjugated

secondary antibodies for 30mins at 37° C in a humidified chamber. The coverslips were washed thrice with PBS and twice with distilled water and mounted onto glass slides. The images were taken using Carl Zeiss LSM510 META confocal microscope.

Statistical Methods

All experiments were performed at least thrice, and data are shown as mean and standard error of mean. Statistical significance was calculated using the GraphPad prism software. To compare two groups the paired Student's t-test was performed. A p-value below 0.05 was considered statistically significant and throughout the p-values have been defined as follows * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, while “ns” represents a non-significant p-value above 0.05.

For the quantification of protein levels from western bolts, the films were scanned and the intensity of bands was measured using ImageJ software. The final relative quantification values are the ratio of net band to net loading control.

Analysis of qPCR array:

Control ΔC_t :

Control target gene C_t value - Control house keeping (Reference) gene C_t value

Test ΔC_t :

Test target gene C_t value – Test house keeping (Reference) gene C_t value

$\Delta\Delta C_t$: Test ΔC_t - Control ΔC_t

Fold change: $2^{(-\Delta\Delta C_t)}$

CHAPTER 3: RESULTS

PART:1

Differential phosphorylation of HPV E6 during the cell cycle

Since phosphorylation plays a major role in influencing E6 substrate specificity, we first wanted to ascertain at what point in the cell cycle E6 is normally phosphorylated. To do this, HPV-18-containing HeLa cells were synchronized using a double thymidine block and, following release, cell extracts were made at different points in the cell cycle, as verified by FACS analysis (Figure 15A). The extracts were then analysed by western blotting using a specific antibody raised against HPV-18 E6 phosphorylated on T156 within the E6 PBM (Boon, Tomaic et al. 2015). The results in Figure 15B demonstrate very low levels of E6 phosphorylation in asynchronously growing cells, with a slight increase in the levels of phospho-E6 in the G1 population, and undetectable levels of phosphorylation of E6 in the S and G2/M phases of the cell cycle. We also performed an analysis of E6 phospho-status in cells that were arrested in G2/M by nocodazole treatment, since previous work from the lab (Boon SS thesis) had indicated G2/M arrest could induce E6 phosphorylation. The results, also shown in Figure 15B, show surprisingly high levels of E6 phosphorylation in these G2/M phase-arrested cells, indicating that nocodazole can also induce E6 phosphorylation. This contrasts with the results from the double thymidine block, where no phosphorylation of E6 was observed in the G2/M population of cells. In order to determine whether there is phosphorylation of E6 when the cells re-enter G1, the assay was repeated and the cells followed for an extended period of time, so that the cells would complete the cell cycle and re-enter into G1 phase. As can be seen from Figure 15C the cells re-enter the G1 phase 12h after release from the double thymidine block. However, in contrast to the cells in the G1-arrested cells, there is no detectable phosphorylation of E6 when the cells re-enter G1. These results suggest that in normal cycling cells the levels of E6 phosphorylation are very low and only following cell growth arrest, albeit when induced through different means, does E6 become phosphorylated. These results suggest that there may be multiple kinases capable of phosphorylating E6, depending upon the precise stimulus.

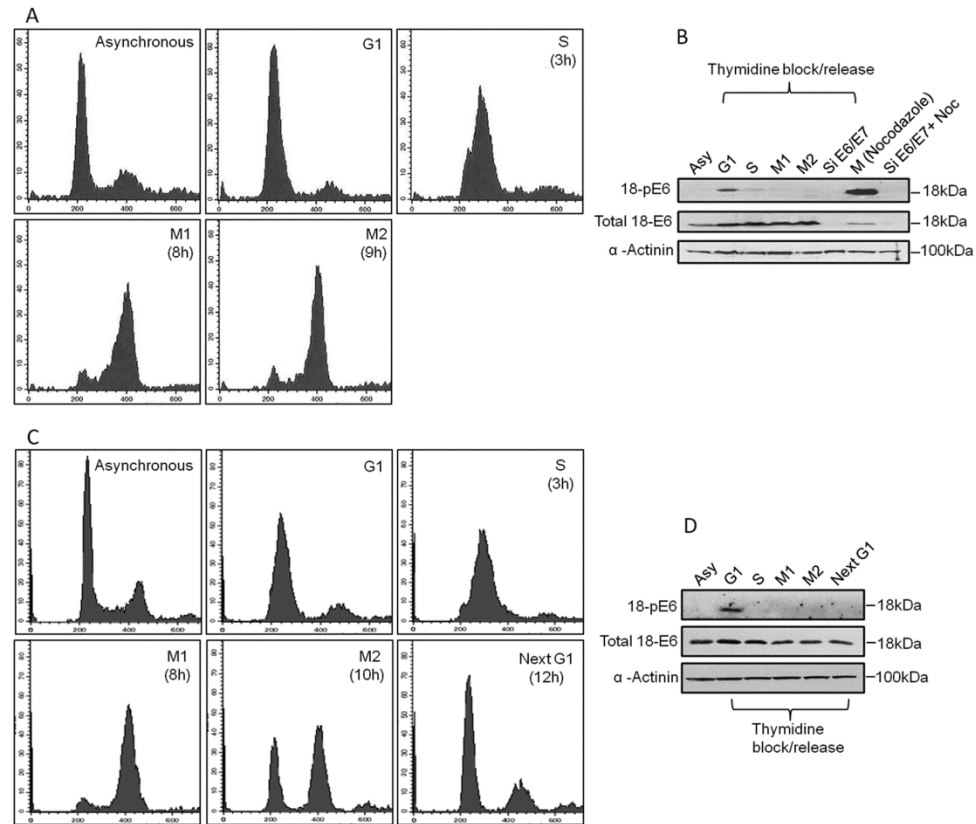


Figure 15. HPV-18 E6 oncoprotein is differentially phosphorylated during the cell cycle. A) FACS analysis of HeLa cells synchronized by double thymidine block and subsequently released and harvested at different times to obtain cells in different phases of the cell cycle: G1=0h, S=3h, M1=8h, M2=9/10h. B) Western blot analysis of HeLa cell extracts from G1, S, M1 and M2 and cells probed with antibodies against phosphorylated E6 (18-pE6) and total E6; α -actinin was used as a loading control. C) FACS analysis of HeLa cells synchronized by double thymidine block and subsequently released and harvested at different times to obtain cells in different phases of the cell cycle: G1=0h, S=3h, M1=8h, M2=9/10h and Next G1=12h. D) Western blot analysis of HeLa cell extracts from G1, S, M1, M2 and next G1. The cells were probed with antibodies against phosphorylated E6 (18-pE6) and total E6; α -actinin was used as a loading control.

We next investigated whether the nocodazole-induced E6 phosphorylation patterns might not, in fact, reflect the cell cycle phase *per se*, but might, instead, be part of a stress response. Indeed, nocodazole has been implicated in induction of oxidative stress (Signoretto, Honisch et al. 2016) and DNA damage in the cell (Dalton, Nandan et al. 2007, Orth, Loewer et al. 2012). One of the potential mechanisms is inhibition of MTH1 (Kawamura, Kawatani et al. 2016), which leads to the incorporation of oxidized nucleotides into the DNA and subsequent DNA damage (Gad, Koolmeister et al. 2014).

To ascertain whether the phosphorylation of E6 following exposure to nocodazole might be an oxidative stress response, we performed a series of analyses of nocodazole-treated HeLa cells, using H₂O₂ treatment as a positive control for the induction of oxidative stress. The E6 phospho-status in these cell extracts was then analysed by western blotting. The results are shown in Figure 16A and demonstrate a strong increase in the levels of E6 phosphorylation in response to H₂O₂ and nocodazole treatment. To determine whether this increase in E6 phosphorylation was due to induction of oxidative stress, we treated the cells with N-acetyl-cysteine (NAC), an antioxidant, following the exposure to H₂O₂ and nocodazole. As can be seen in Figure 16A, H₂O₂ and nocodazole-induced E6 phosphorylation were significantly reduced upon treatment with NAC, suggesting an involvement of oxidative stress-response kinases in E6 phosphorylation.

Interestingly, we also found that blocking protein synthesis using cycloheximide (CHX) results in significantly elevated levels of phospho-E6, although, as expected, the total level of E6 is reduced under these conditions (Figure 16A). However, in this case, NAC did not reduce the phospho-E6 levels, suggesting that the cycloheximide-induced phosphorylation of E6 is not an oxidative stress response, but rather that another stress-response pathway(s) might be involved.

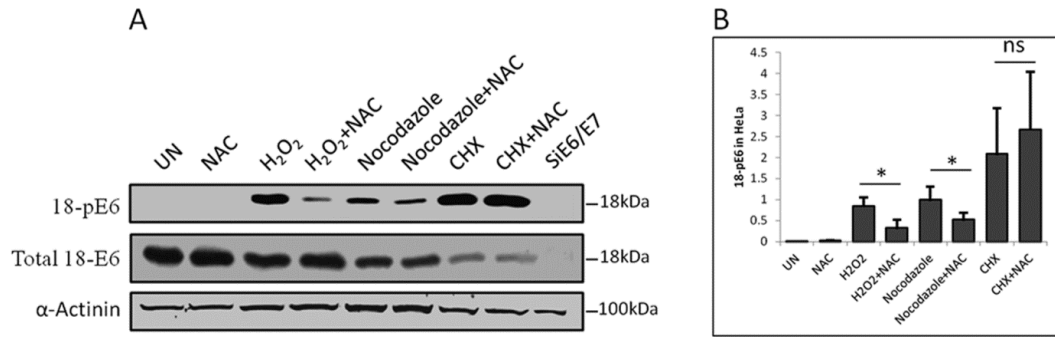


Figure 16. HPV-18 E6 is phosphorylated as a result of cellular stress responses.

A) Representative western blot analysis of HeLa cells treated with H₂O₂ for 4hrs, Nocodazole for 15 hrs or cycloheximide (CHX) for 4hrs, in the presence or absence of the antioxidant N-acetyl-cysteine (NAC). Blots were probed with antibodies against total HPV-18 E6 and HPV-18 E6 phospho T156; α -actinin was used as a loading control. B) The histogram shows the quantification of pE6 normalized to actinin from at least three independent experiments, statistically quantified by using Student's t-test; standard error of the mean is shown. P value: *= 0.05; ns=non significant.

Potential involvement of DNA Damage Response (DDR) kinases in regulation of E6-phosphorylation

The above results suggest that one source of signaling through oxidative stress could be through stimulation of a DDR. Accordingly, we compared the known amino acid recognition motifs of a number of DDR kinases, including ATM, ATR, Chk1 and Chk2, with the sequence of the HPV-18 E6 PBM. As can be seen from Figure 17A, HPV-18 E6 has a good match with the consensus site for Chk1. In order to determine whether E6 could be phosphorylated by either Chk1 or Chk2, we performed an *in vitro* kinase assay using the purified kinases with purified HPV-18 E6 GST fusion protein in the presence of radiolabeled [γ - 32 P] ATP. The results in Figure 17B demonstrate that HPV-18 E6 is a very good substrate for phosphorylation by Chk1, but not by Chk2.

In order to verify whether the phospho-acceptor site was T156 embedded within the E6 PBM, we also included the T156E mutant in the phosphorylation assays. The results are also shown in Figure 17B and demonstrate that the T156E mutant abolishes the phosphorylation of E6 by Chk1, indicating that the T156E residue is the primary phospho-acceptor site in HPV-18 E6. To ascertain whether any of these DDR kinases might indeed be involved in stress-responsive E6-phosphorylation *in vivo*, we treated HeLa cells with specific inhibitors of ATR, ATM, Chk1 or Chk2 kinases for 15hrs and then exposed the cells to either nocodazole or H₂O₂. The results obtained are shown in Figure 18 and demonstrate that under non-stressed conditions the levels of E6 phosphorylation are, again, very low. Following exposure to nocodazole, E6 is clearly phosphorylated, but this is greatly decreased following inhibition of the Chk1 kinase, suggesting that this is responsible for phosphorylating E6 in response to nocodazole treatment (Figure 18B). In contrast, in the case of H₂O₂, the greatest inhibition of phosphorylation occurs following treatment with the Chk2 inhibitor (Figure 18C). This was rather surprising considering the low level of phosphorylation of E6 by Chk2 observed *in vitro* in Figure 17. However, previous studies had indicated that one downstream kinase activated by DDR kinases was

PKA (Searle, Schollaert et al. 2004, Bensimon, Aebersold et al. 2011, Marazita, Ogara et al. 2012). Since we had previously shown that HPV-18 E6 is a very good substrate for PKA *in vitro* (Kuhne, Gardiol et al. 2000, Boon and Banks 2013, Boon, Tomaic et al. 2015), we repeated the analysis but also treated cells with H89 to block PKA activity in the H₂O₂ exposed cells. The results in Figure 18D show a marked inhibition of H₂O₂-induced phosphorylation of E6 following the inhibition of either PKA or Chk2. These results indicate that PKA is most likely responsible for phosphorylating E6 following the activation of Chk2 in response to an H₂O₂ oxidative stress-induced DDR. In order to further confirm the Chk2 activation after exposure to H₂O₂, we analysed the levels of phosphorylated Chk2 (Thr 68). Indeed, H₂O₂ treatment leads to a significant increase in pChk2 levels, and inhibition of ATM and ATR also reduced pChk2 levels. However there was a striking increase in the E6 phosphorylation when ATM and Chk1 were inhibited (Figure 18C, D and E). In order to investigate whether this increase in E6 phosphorylation following inhibition of ATM and Chk1 was also mediated by PKA, the assay was repeated in the presence of the PKA inhibitor H89. The results in Figure 18E demonstrate that increased phosphorylation of E6 following the inhibition of ATM or Chk1 is mediated largely by PKA. This observation supports previous studies showing that Chk1 inhibition causes replication stress and activates the ATR pathway (Gagou, Zuazua-Villar et al. 2010, Petermann, Orta et al. 2010, Choi, Toledo et al. 2011) and that ATR signals activate Chk2 in response to H₂O₂ treatment (Zhang, Gao et al. 2014), which subsequently activates PKA (Bensimon, Aebersold et al. 2011). To justify the specificity and efficacy of the inhibitors, we analysed H₂O₂-induced phospho-Chk1 and phospho-Chk2 levels, which are ATR and ATM substrates respectively. As can be seen in Figure 18E, inhibition of only ATR and ATM resulted in reduced levels of p-Chk2, as both of these kinases can directly phosphorylate Chk2 in response to H₂O₂-induced DDR. Similarly, only inhibition of ATR abolished phosphorylation of Chk1 (Figure 18F), whilst other inhibitors did not affect the p-Chk1 levels in response to H₂O₂ treatment. This supports the good specificity and efficacy of these inhibitors in our assays.

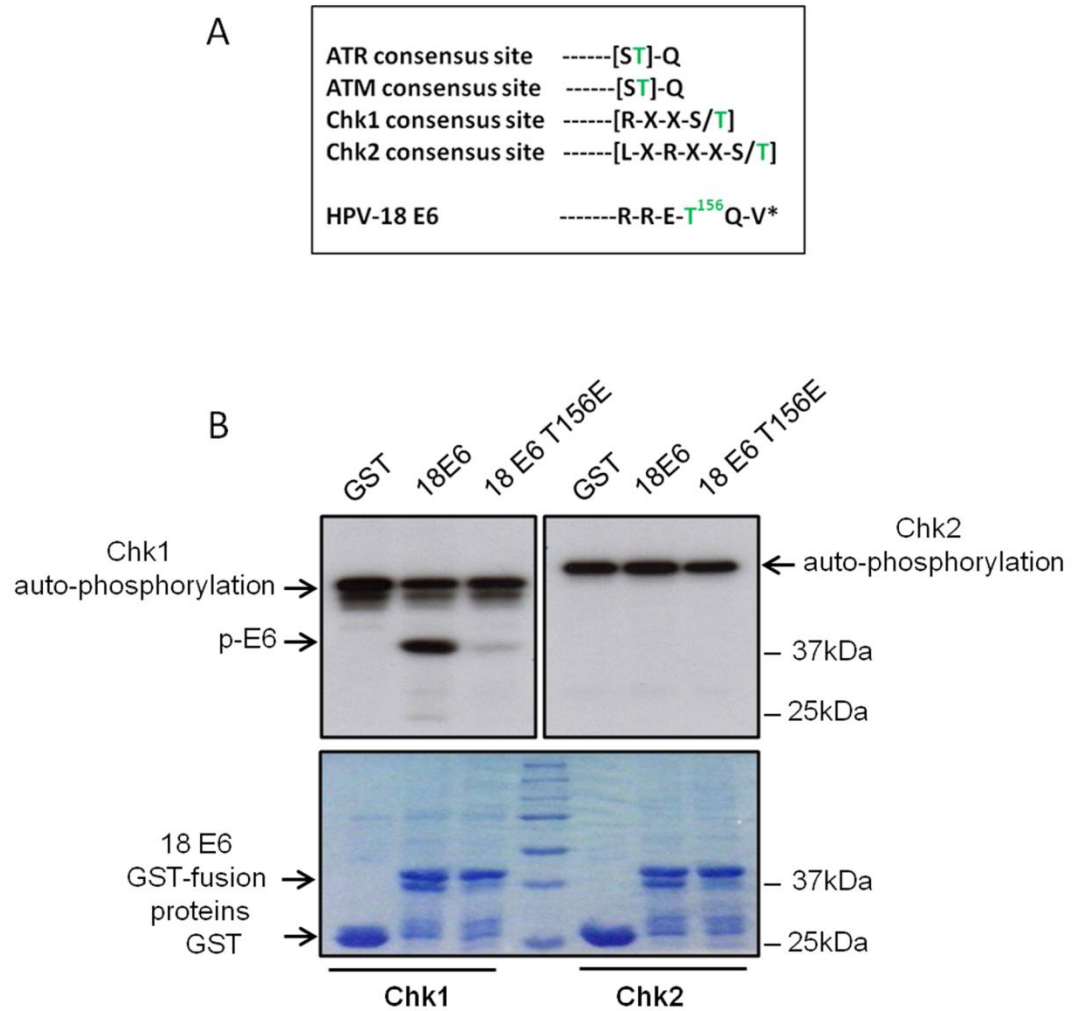
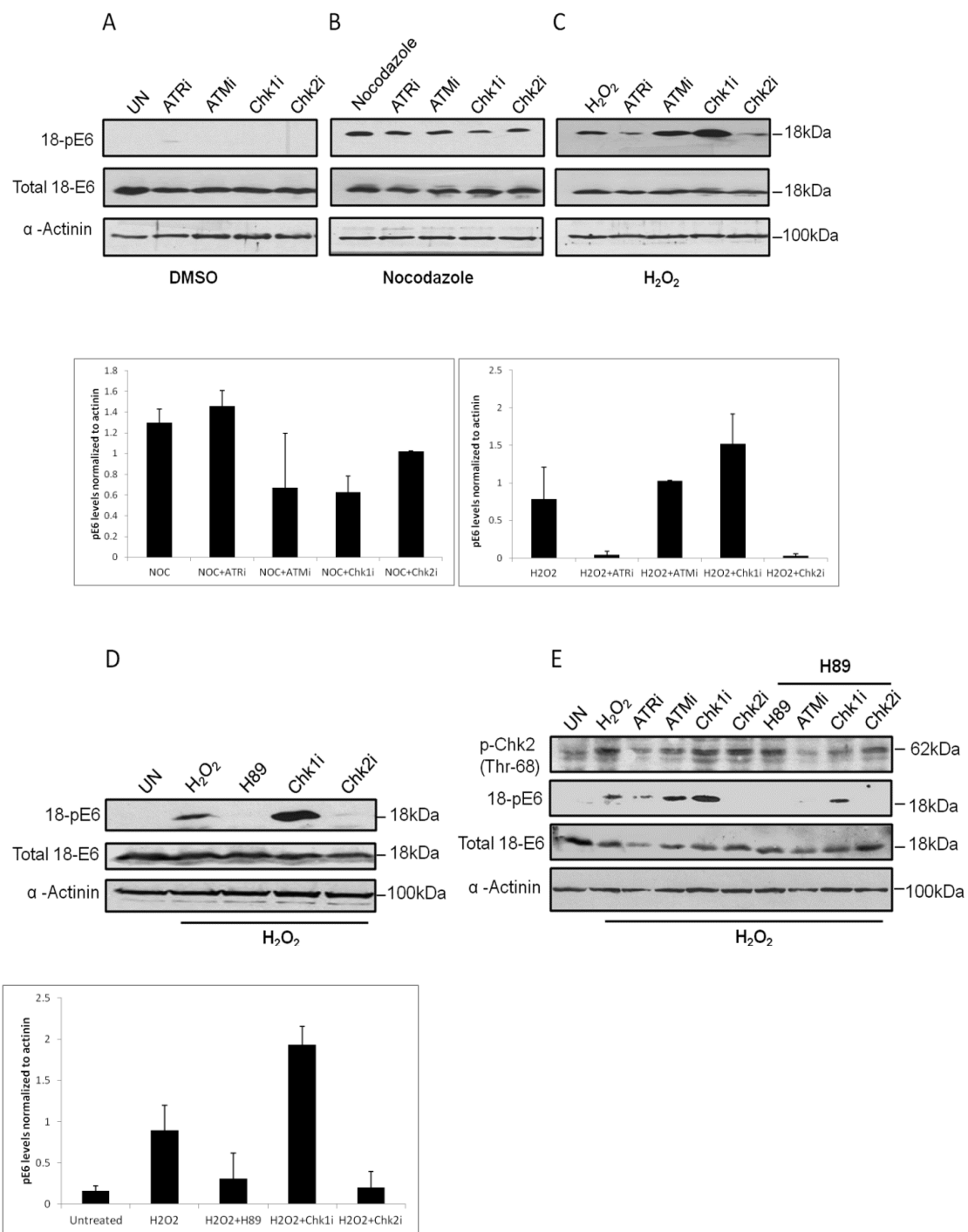


Figure 17. HPV-18 E6 is phosphorylated by the DNA damage response kinase Chk1. A) The consensus phosphorylation motifs recognized by ATR, ATM, Chk1 and Chk2 are compared with the sequence of the carboxy-terminus of HPV-18 E6. B) *In vitro* phosphorylation assay using purified GST fusion proteins of wild type HPV-18 E6 and the HPV-18 E6 T156E mutant incubated with Chk1 or Chk2 in the presence of [γ^{32} P]-ATP. Upper panels show the autoradiograms and the lower panels show the Coomassie-stained SDS PAGE gels. Arrows indicate the phospho E6, the GST E6 fusion protein, the GST control and the auto-phosphorylated Chk1 and Chk2.



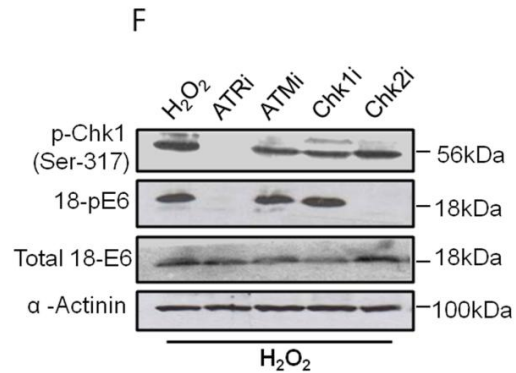


Figure 18. Different DDR response pathways lead to phosphorylation of E6 in response to different stresses.

A-C) HeLa cells were treated with specific inhibitors of ATR, ATM, Chk1 or Chk2 for 15h, then treated with DMSO as a control (A) or treated with Nocodazole (B) or H₂O₂ (C). Western blots were probed with antibodies against total HPV-18 E6 and HPV-18 E6 phospho T156. α-actinin was used as a loading control. D) The assay was repeated with H₂O₂, additionally treating cells with a specific PKA inhibitor, H89. The pE6 levels were detected in HeLa cells in the presence or absence of PKA inhibitor (H 89), as well as Chk1i and Chk2i. E) HeLa cells were treated with H₂O₂, together with a specific PKA inhibitor, H89, and DDR kinase inhibitors as indicated. The pChk2 levels were analysed to check the efficacy of ATMi and ATRi, and pE6 levels were also analysed in the presence or absence of the various inhibitors. F) HeLa cells were treated with H₂O₂ together with specific DDR kinase inhibitors as indicated. The pChk1 levels were analysed to check the efficacy of DDR inhibitors. The histograms show the quantification of levels of pE6 from respective western blots as indicated, normalized to actinin.

Having shown that DDR response kinases play an important role in regulating E6 phosphorylation, we were interested to know whether ‘DDR-modulators’ might also affect E6-phosphorylation. Since various chemotherapeutic agents have been shown to indirectly modulate the DDR pathways (Cossar, Schache et al. 2017), it was of interest to know what effect they might have on E6 phosphorylation. We used various chemotherapeutic agents that are known to target molecules involved in the DNA replication and DNA repair pathways, as listed in Figure 19A. HeLa cells were exposed to the different chemotherapeutic drugs, and the cells were then harvested and the levels of E6 phosphorylation ascertained by western blotting. At the same time, the levels of p53 expression were also analysed. The results of the analysis are shown in Figure 19B and demonstrate a number of interesting points. Inhibition of topoisomerase, ribonucleotide reductase and DNA replication all provoke a pronounced increase in the levels of E6 phosphorylation, suggesting that multiple DDRs can signal to E6. In contrast, PARP inhibitors have been documented to induce double-strand DNA breaks, resulting in the accumulation of DNA damage in the cell (Booth, Cruickshanks et al. 2013, Jelinic and Levine 2014). We used three different PARP inhibitors, but none of them induced E6 phosphorylation in HeLa cells, and this appeared to be related to the very low level of DDR activation that was induced in these cells following PARP inhibition, compared with H₂O₂ treatment, as determined by analysing the p-Chk1 and γ -H2AX levels (Figure 19C). Finally, none of the drugs induced an increase in p53 levels, consistent with E6’s ability to target p53 for degradation (Scheffner, Werness et al. 1990, Scheffner, Huibregtse et al. 1993) and, in a subset of cases, p53 levels were also further reduced. This is consistent with previous studies showing that induction of certain DDR pathways can also increase the levels of E6-induced p53 degradation (Tomaic, Pim et al. 2011).

Having found that multiple DDR-inducing agents could increase the levels of E6 phosphorylation, we then wanted to determine whether Chk1 or Chk2 was responsible. To do this we chose to analyse cisplatin: HeLa cells were exposed to cisplatin in the presence of different DDR kinase inhibitors. The cells were then

harvested and the levels of E6 phosphorylation were analysed by western blotting. The results in Figure 19D demonstrate that the majority of E6 phosphorylation that occurs following exposure to cisplatin is Chk1-dependent.

I also observed that the total E6 levels are very low in the presence of various DNA damage inducing agents. In order to determine whether a similar induction of E6 phosphorylation takes place in the cells other than HeLa, we proceeded to repeat the assays in C4-1 cells which are also HPV-18 positive cervical cancer derived cells. As can be seen in Figure 20, both teniposide and etoposide are able to induce a strong phosphorylation of E6, whereas cisplatin-induced pE6 levels in C4-1 cells were relatively low, compared with those in HeLa cells. This could be presumably due to differences in the activation of DDR pathway in different cell lines. The specificity of anti-18-pE6 antibody was tested in NIKS and NIKS stably expressing 16E6 and 16E6 Δ PBM, in the presence of Etoposide together with or without CBZ. HeLa cells treated with Etoposide or cisplatin were used as positive control for antibody. Our results demonstrate that the 18-pE6 antibody is highly specific (Figure 20C).

Taken together, the above results indicate that various forms of DDR result in phosphorylation of E6. This can be mediated by Chk1 in response to an oxidative stress response following treatment with nocodazole, and in response to a DDR following treatment with cisplatin. However, in the case of an oxidative stress response induced by H₂O₂, the phosphorylation of E6 occurs through a Chk2-activated pathway that requires downstream PKA to phosphorylate E6 directly.

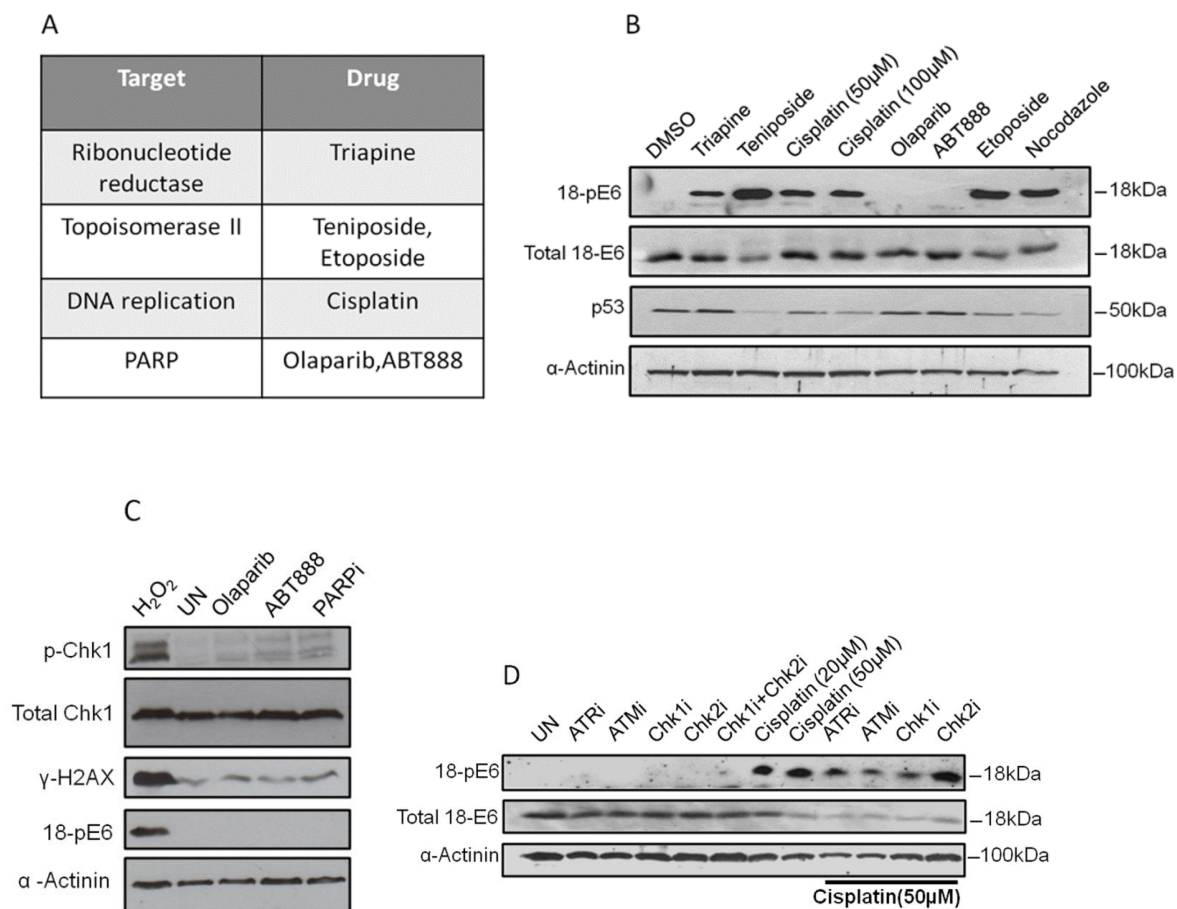


Figure 19. Exposure to DNA damage-inducing chemotherapeutic agents can induce E6 phosphorylation.

A) Table showing the chemotherapeutic agents used and their cellular targets. B) HeLa cells were treated with the indicated drugs for 15h prior to harvesting and analyzing by western blot probed with antibodies against total HPV-18 E6, HPV-18 pE6 and p53; α -actinin was used as a loading control. C) HeLa cells were either untreated or treated with H_2O_2 for 4hrs or with the indicated PARP inhibitors for 15h, and the levels of pChk1, total Chk1, γ -H₂AX and HPV-18 pE6 were analysed by western blotting. The α -actinin was used as a loading control. D) The assay was repeated with cisplatin, additionally including specific kinase inhibitors, and the western blot was probed with antibodies against total HPV-18 E6, HPV-18 pE6 and α -actinin was used as a loading control.

Previous studies have reported that phosphorylation of E6 within the PBM results in inhibition of the PDZ interaction (Kuhne, Gardiol et al. 2000, Boon and Banks 2013, Boon, Tomaic et al. 2015). Therefore, the prediction from these studies is that the increased phosphorylation of E6 induced by DDR kinases should result in the rescue of certain PDZ domain containing substrates of E6 from proteasome mediated degradation. To investigate this, we treated HeLa cells with Triapine or cisplatin and assessed the levels of expression of Dlg. As a positive control, we transfected the cells with siRNA against E6/E7. The results in Figure 21 show that the ablation of E6 results in increase in the levels of Dlg protein, which is consistent with previous studies. Interestingly, treatment with Triapine or cisplatin also rescues the levels of Dlg protein expression to a degree which reflects the induction of phosphorylation of E6.

Taken together, the results demonstrate that a functional consequence of DDR kinase mediated E6 phosphorylation is rescue of PDZ domain containing substrate of E6 from E6-induced degradation, consistent with the phosphorylation event making E6 incapable of binding PDZ domains.

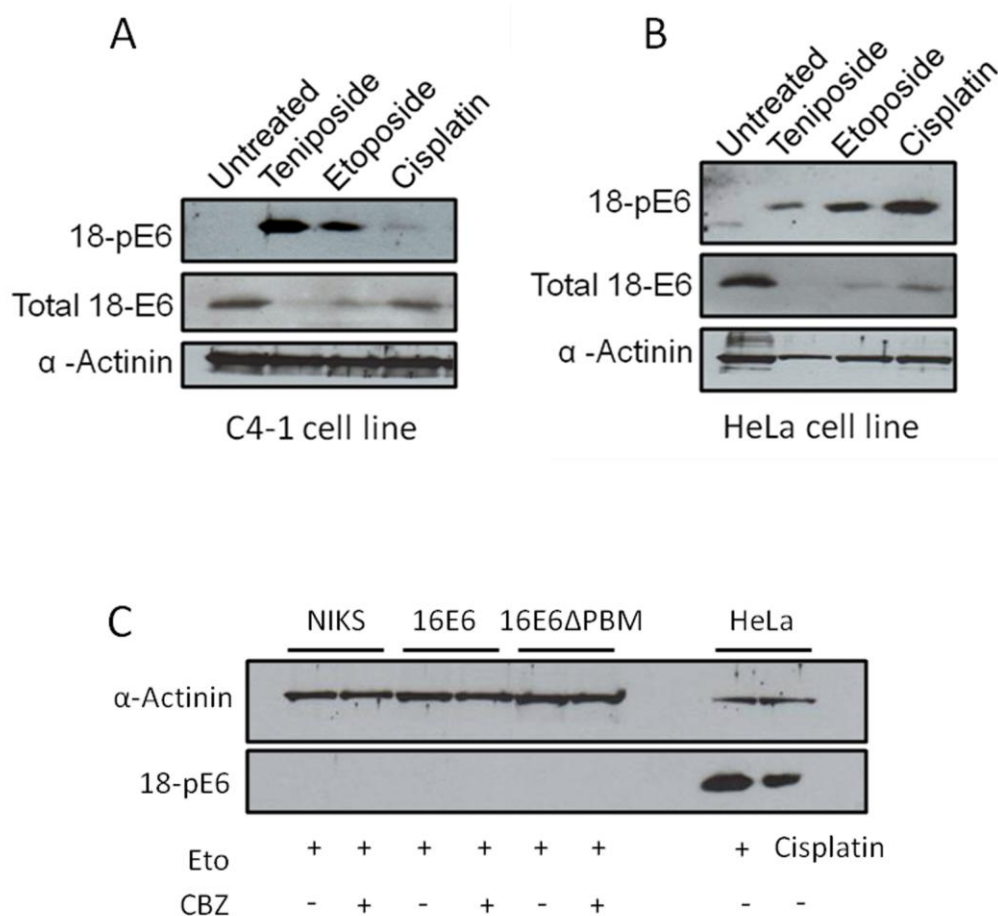


Figure 20. E6 is susceptible to phosphorylation upon treatment with chemotherapeutic drugs in the C4-1 cervical cancer cell line.

C4-1 (A) and HeLa (B) cells were treated with the indicated chemotherapeutic drugs for 15h. Cell extracts were then analyzed by western blot probed with antibodies against total HPV-18 E6 and HPV-18 pE6; α -actinin was used as a loading control. C) The specificity of anti-18-pE6 antibody was tested in NIKS, 16E6 and 16E6 Δ PBM NIKS in the presence or absence of Etoposide and CBZ as indicated. HeLa cells treated with Etoposide or cisplatin were used as positive control for antibody.

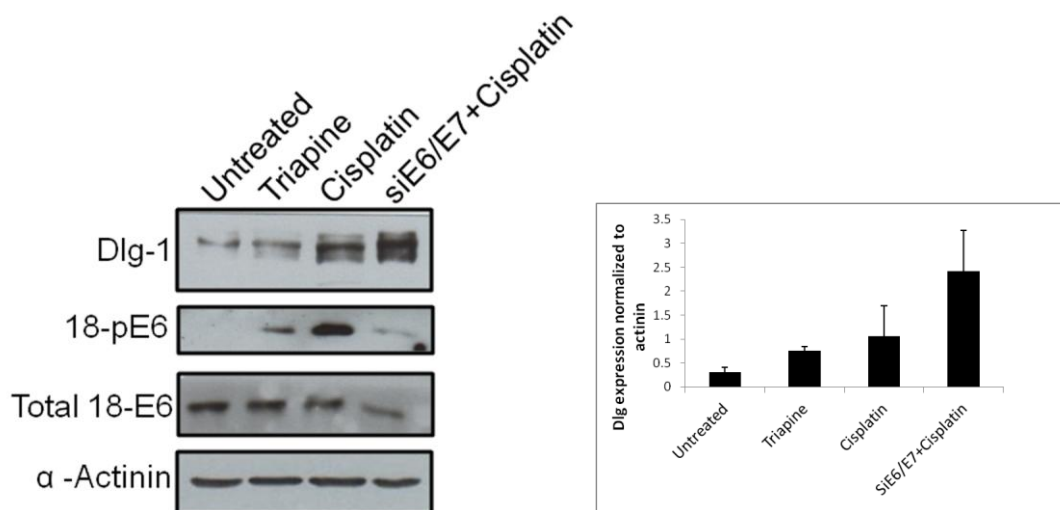


Figure 21. Phosphorylation of E6 rescues Dlg1 expression in HeLa cells.

HeLa cells were treated either with the indicated chemotherapeutic drugs, or with siRNA against E6/E7 plus cisplatin for 15h. Cell extracts were analyzed by western blot probed with antibodies against Dlg1, total HPV-18 E6 and HPV-18 pE6; α -actinin was used as a loading control. The histogram shows quantification of Dlg expression normalized to actinin control.

The HPV-18 E6 PBM perturbs p53 transcriptional transactivation activity

Having shown that the HPV E6 PBM can be phosphorylated by a number of stress-response kinases in response to various cellular stresses, the next question was: what is the biological result of the E6 phosphorylation? Interestingly, a number of recent studies with HPV-16 and HPV-18 in organotypic systems have shown that the E6 PBM is required for viral genome episomal maintenance and, furthermore, that p53 loss can partly rescue this episome loss following the mutation of the E6 PBM (Lorenz, Rivera Cardona et al. 2013, Brimer and Vande Pol 2014). It is also known that HPVs activate the ATM/ATR DNA repair pathway and that this is necessary for HPV genome amplification (Moody and Laimins 2009, Reinson, Toots et al. 2013, Hong, Cheng et al. 2015). Taken together, these studies suggest a probable link between the phosphorylation of the E6 PBM and perturbation of p53 activity. One potential common link between these observations is 14-3-3, since this family of proteins is linked to the regulation of p53 transcriptional transactivation activity during cell cycle check-point control (Waterman, Shenk et al. 1995, Waterman, Stavridi et al. 1998, Rajagopalan, Jaulent et al. 2008).

Therefore, we initiated a series of studies to investigate whether the E6 PBM has any effect on p53's transcriptional transactivation of a variety of different promoters: PUMA, p21, Mdm2 and BAX, using Renilla luciferase as a control of transfection efficiency. Since a major function of HPV-18 E6 is the degradation of p53 through the ubiquitin proteasome pathway (Scheffner, Werness et al. 1990, Scheffner, Huibregtse et al. 1993), it was necessary to perform our studies in the presence of the proteasome inhibitor, CBZ (MG132). The p53-null H1299 cells were transfected with the appropriate reporter constructs, together with p53 and different HPV-18 E6 expression plasmids. In the first analysis, we focused on wild type HPV-18 E6 and the HPV-18 E6 Δ PBM mutant. After 24hrs, cells were harvested and luciferase activity was measured using the dual-luciferase assay system, and the results are shown in Figure 22. As can be seen, there are some striking differences between these promoters in how the E6 PBM can modulate p53 transcriptional activity

following proteasome inhibition. In all cases, in the absence of proteasome inhibition the wild type and E6 Δ PBM mutant show similar abilities to inhibit p53 transcriptional activity. Following proteasome inhibition, the wild type E6 retains the ability to inhibit p53 transcriptional activity. In contrast, for the E6 Δ PBM mutant this only holds true for the BAX promoter (Figure 22C), where both wild type and mutant E6 retain inhibition of p53 transcriptional activity in the presence of proteasome inhibitors. In the case of p21 (Figure 22A), the E6 Δ PBM allows a modest increase in p53 transcriptional activity, but this is even more apparent with PUMA (Figure 22B) and Mdm2 (Figure 22D) where lack of a PBM allows a marked increase in the level of p53 transcriptional activity in the presence of proteasome inhibitors.

In order to elucidate whether this effect was linked to the ability of E6 to be phosphorylated, and not due to an overall defect in PDZ interaction, we included the HPV-18 E6 R153G mutant, which we have shown previously to be defective in the phospho-recognition motif for PKA (Boon and Banks 2013) and for Chk1 (See Figure 28B), but which retains PDZ binding potential. The results on the Mdm2 promoter are shown in Figure 22D and demonstrate that it has similar ability to block p53 transcription as the wild type E6, but that following addition of proteasome inhibitors this activity is compromised and there is a dramatic increase in p53 transcriptional activity, comparable to that seen with the E6 Δ PBM mutant.

Taken together, these results demonstrate that HPV-18 E6 can inhibit p53 transcriptional activity in a manner that is PBM and phospho-site dependent, indicating that phosphorylation of E6 within the PBM in response to a variety of DDR signals can directly link PBM function to the inhibition of p53 transcriptional activity.

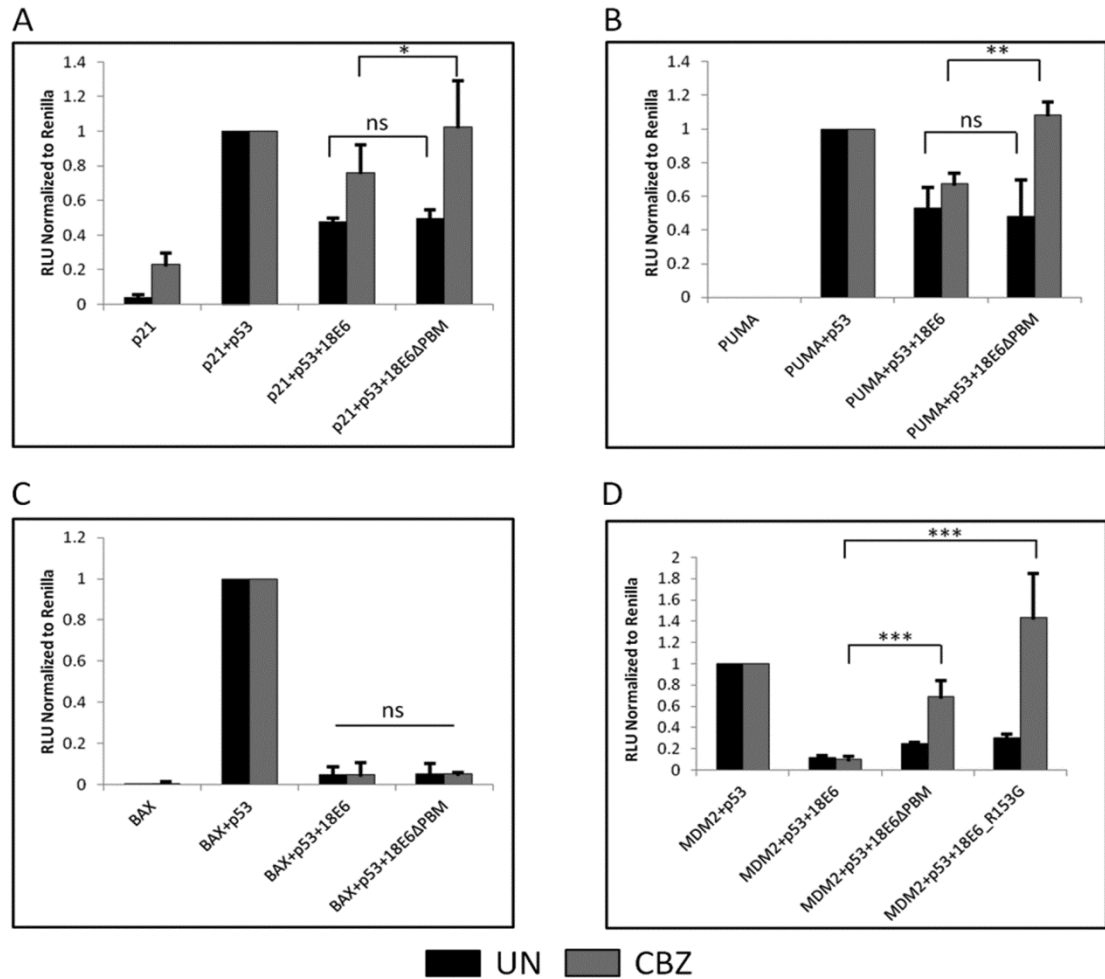


Figure 22. The HPV-18 E6 PBM contributes towards inhibition of p53 transcriptional transactivation activity.

H1299 cells were transfected with the indicated promoter constructs upstream of a luciferase promoter (A: p21-Luc, B: PUMA-Luc, C: BAX-Luc, D: Mdm2-Luc), together with p53 and either wild type or mutant HPV-18 E6 in the presence or absence of the proteasome inhibitor CBZ. The histograms show the results from at least three independent experiments, quantified using Student's t-test and the standard error of mean is shown. Also shown are the p values (* <0.05, ** < 0.005, *** <0.0005, ns=non significant) for the changes in the relative luciferase activity.

We next wanted to investigate how many other p53 responsive genes might be similarly regulated. But we wanted to perform our assays in a more relevant biological setting and to do this, we decided to focus on NIKS (Normal Immortalized Keratinocytes), also stably expressing wild type 16E6 or the 16E6 Δ PBM mutant.

In the next set of assays, we wanted to monitor p53-mediated induction of p21 in the NIKS also stably expressing wild type 16E6 or 16E6 Δ PBM mutant in the presence or absence of DNA damaging agents or proteasome inhibitor. In order to ascertain whether there was similar effect as can be seen in the p21 promoter transcriptional transactivation in H1299 cells (Figure 23A), NIKS were treated with Etoposide and Thymidine to induce p53 DDR in the presence or absence of CBZ. The levels of p53 and p21 were then analysed after 24h by western blotting. The results in Figure 23B show that the p21 expression is elevated in 16E6 Δ PBM mutant as compared to NIKS expressing wild type 16E6, despite the levels of p53 being equal in both the cell lines.

Having found that the PBM could affect the p53 transcriptional activity also in NIKS cells, we then wanted to determine whether the increased p53 transcriptional activity seen with the 16E6 Δ PBM mutant was due to 14-3-3. To do this, the assay was repeated in the presence of the pan 14-3-3 inhibitor, Difoepin. The results are shown in Figure 24A and demonstrate that there is a modest reduction in the levels of p21 protein expression in the presence of pan 14-3-3 inhibitor. This suggest that 14-3-3 is important for p53 induction of p21, which is in agreement with previous studies (Rajagopalan, Jaulent et al. 2008), and also provides a mechanism whereby the E6 PBM interaction with 14-3-3 might inhibit 14-3-3 regulation of p53 transcriptional activity.

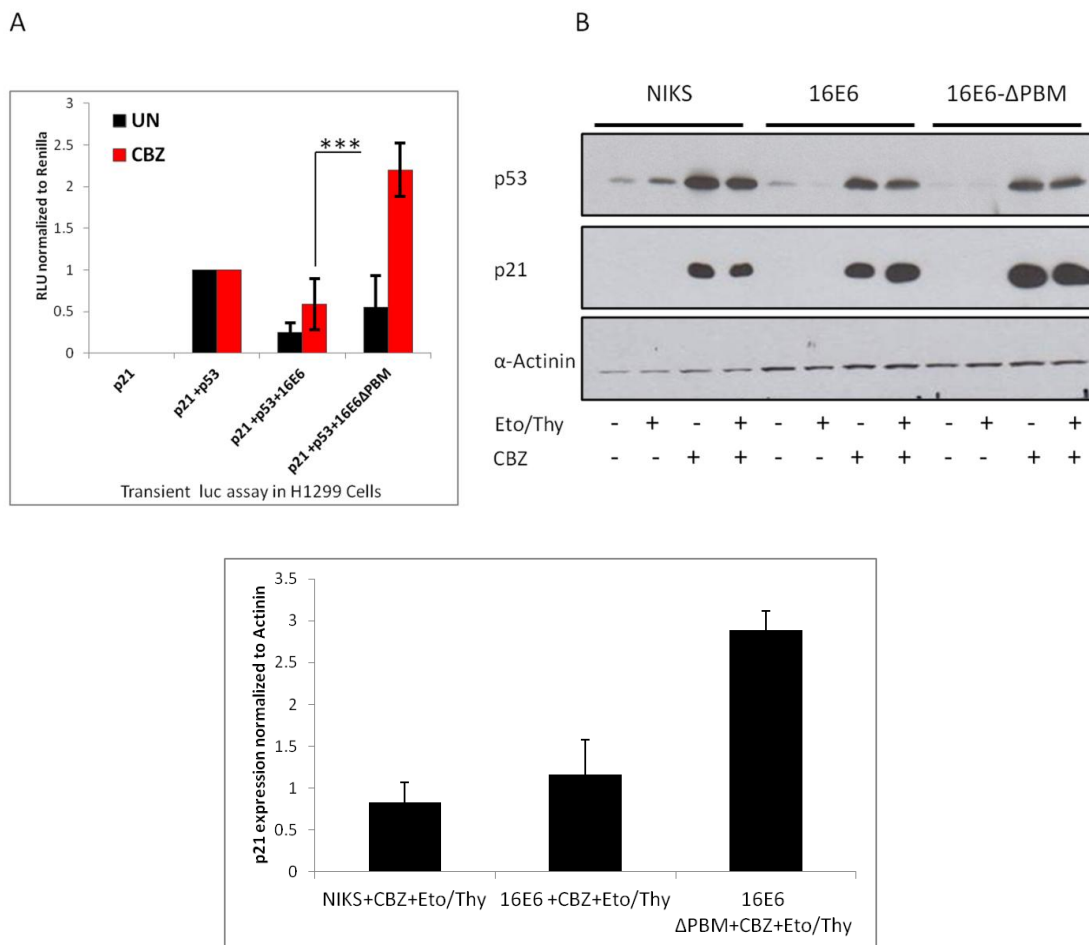


Figure 23. E6's inactivation of p53 is mediated through the PBM.

A) H1299 cells were co-transfected with plasmids expressing the Luciferase reporter gene promoter p21-Luc and FLAG- p53, together with either HPV-16 E6 wild type or ΔPBM mutant in the presence or absence of CBZ. The histogram represents the mean relative luciferase readings from at least three independent experiments, and standard deviation is shown. Also shown is the p value (significance value: ≤ 0.05) of the changes of the relative luciferase activity. B) Western blot analysis of p53 responsive gene, p21, in NIKS, 16E6 NIKS and 16E6 ΔPBM NIKS upon DNA damage-induced p53 transcriptional trans-activation, in the presence or absence of proteasome inhibitor (CBZ). Eto: Etoposide, Thy: Thymidine. The histogram shows quantification of p21 expression normalized to actinin from at least 3 independent western blot experiments.

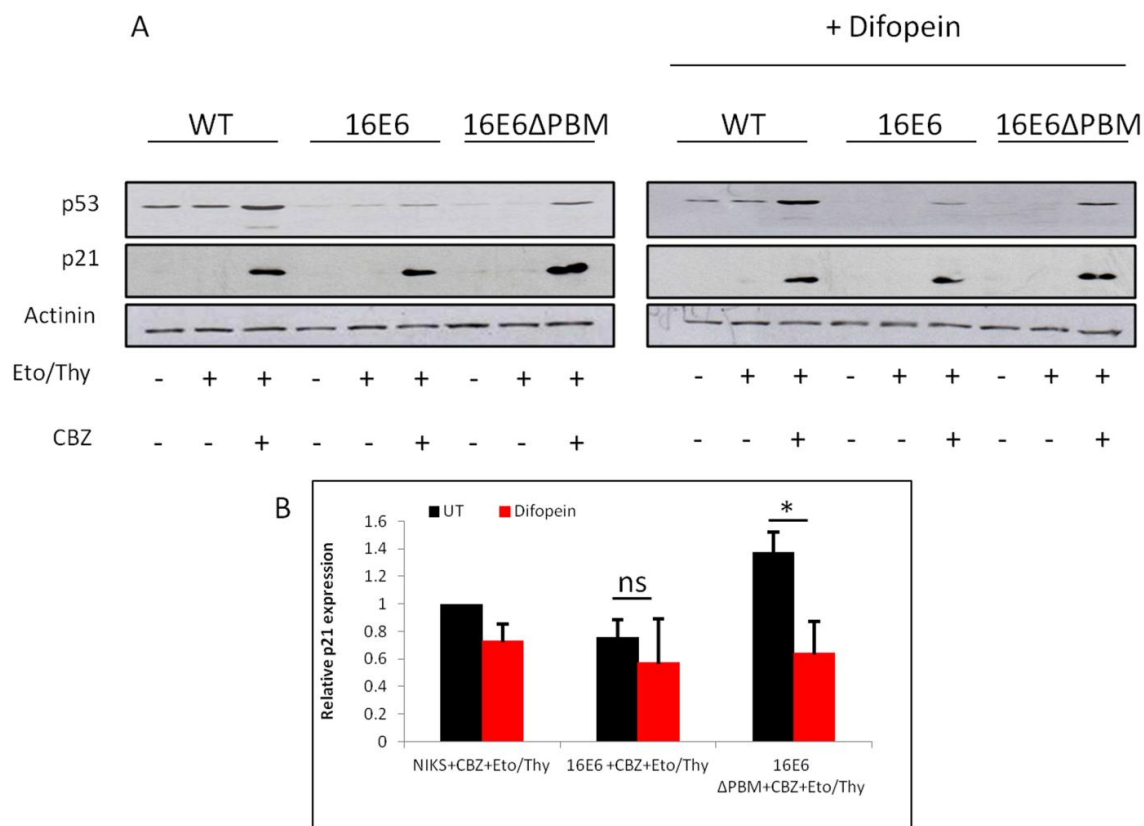


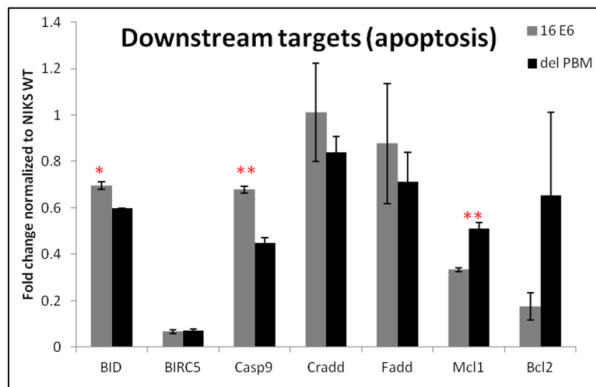
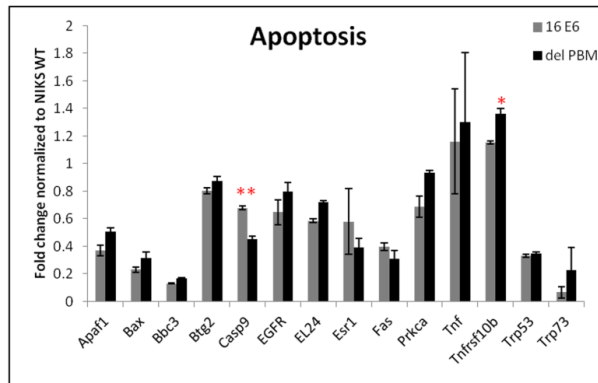
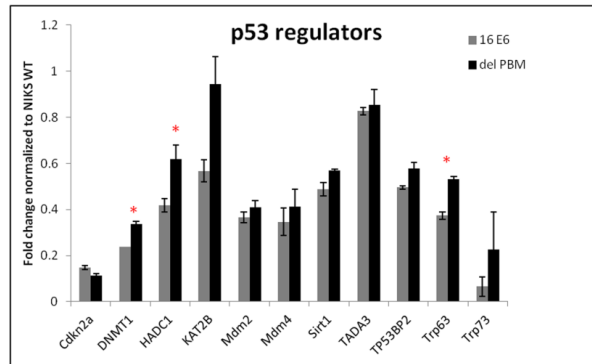
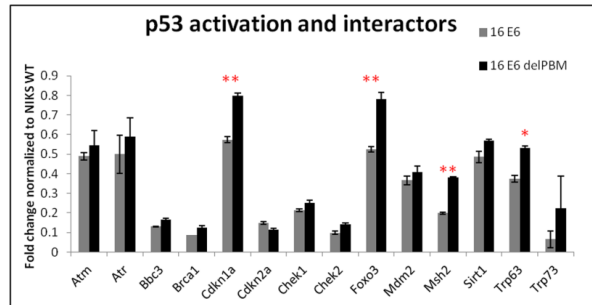
Figure 24. Inhibition of 14-3-3 alters E6 PBM-mediated p53 inactivation.

A) Western blot analysis of p53 responsive gene, p21, in NIKS, 16E6 NIKS and 16E6 ΔPBM NIKS upon DNA damage-induced p53 transcriptional trans-activation, in the presence or absence of proteasome inhibitor (CBZ). B) The histogram shows the results from at least three independent experiments, statistically quantified by using Student's t-test; standard error of the mean is shown. P value: *= 0.05; ns=non significant. UT: Untreated, Eto: Etoposide, Thy: Thymidine.

We then proceeded to investigate in more detail which other p53 responsive genes were similarly regulated in a E6 PBM dependent manner. To do this, we performed experiments using a p53 RT PCR array. This array covers 84 genes, which are involved in: 1. p53 Activation, 2. p53 regulation, 3. p53 interactions, 4. Apoptosis, 5. Cell cycle and 6. DNA damage response.

For the array analysis, we performed this in NIKS, 16E6 NIKS and 16E6 Δ PBM NIKS. The cells were treated with DNA damaging agent, etoposide and proteasome inhibitor, as before. The real-time PCR data was normalized with the GAPDH housekeeping gene. To calculate the fold upregulation or downregulation at the mRNA level, the values from the 16E6 NIKS and 16E6 Δ PBM NIKS were normalized to NIKS, and the data is shown in histograms in Figure 25. Of the 84 genes assessed, 19 were seen to be upregulated in 16E6 Δ PBM NIKS compared with 16E6 NIKS, whilst only 3 were downregulated. Eleven of the 84 genes are direct targets of p53, of which four (Bbc3, Ccng1, Cdkn1a and Tnfrsf10b) were upregulated in 16E6 Δ PBM NIKS cells, compared with 16E6 NIKS.

We also validated some of these targets at the protein level by western blot analysis and, as can be seen in Figure 13, Mcl-1, p21, p63 and E2F1 were regulated in an E6 PBM- and 14-3-3-dependent manner. In agreement with the data from the mRNA analysis, the expression levels of PCNA were also elevated in 16E6 Δ PBM NIKS, compared with 16E6 NIKS; however, inhibition of 14-3-3 did not change these levels, suggesting that PCNA is regulated in a PBM-dependent, but independent from 14-3-3. We also tested the levels of Cyclin-B1 and EGFR, which showed significant differences at the mRNA level, but not at the protein level. This analysis suggests that E6, through its PBM, regulates various p53 responsive genes, as well as some of its interacting partners and regulators (Figure 26).



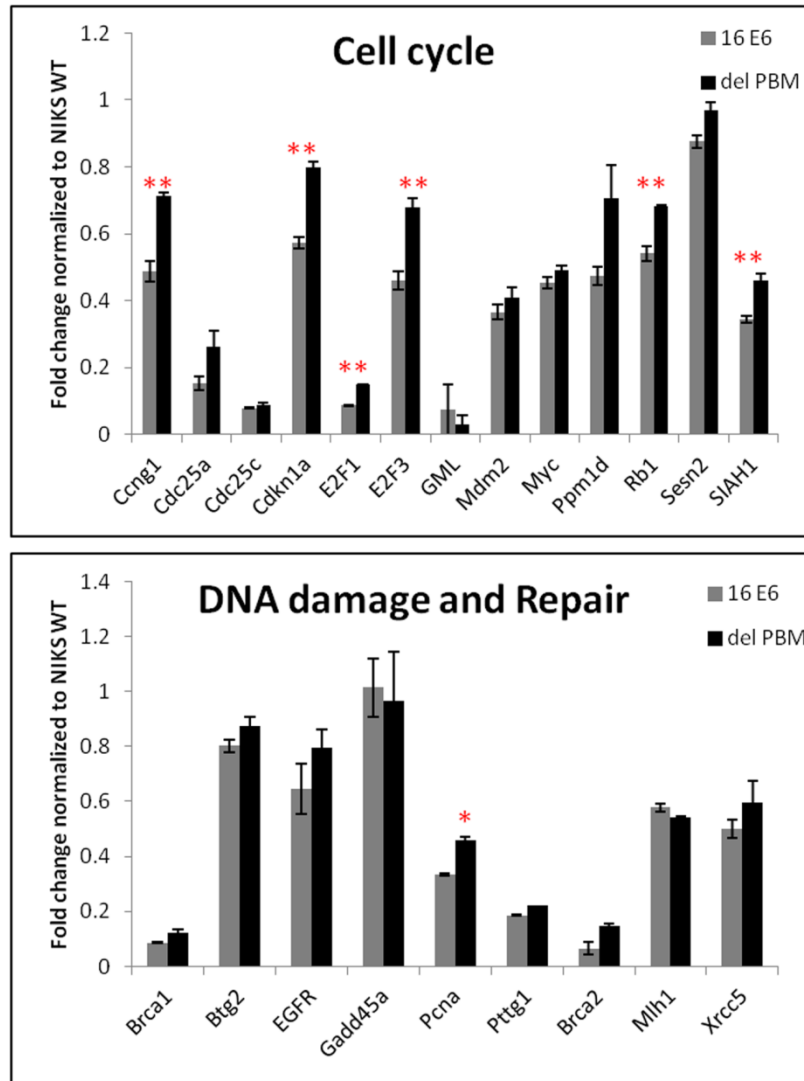


Figure 25. Analysis of p53 signaling in NIKS-16E6 and NIKS-16E6 Δ PBM by RT PCR array.

The histograms show the results from two independent experiments. The $\Delta\Delta C_t$ values were obtained by normalizing the C_t values of genes from NIKS-16E6 and NIKS-16E6 Δ PBM cell lines with those of NIKS cells. GAPDH was used as housekeeping gene to normalize the data. Student's t-test was used for statistical quantification; standard error of the mean is shown. P value: *= 0.05, **=0.005.

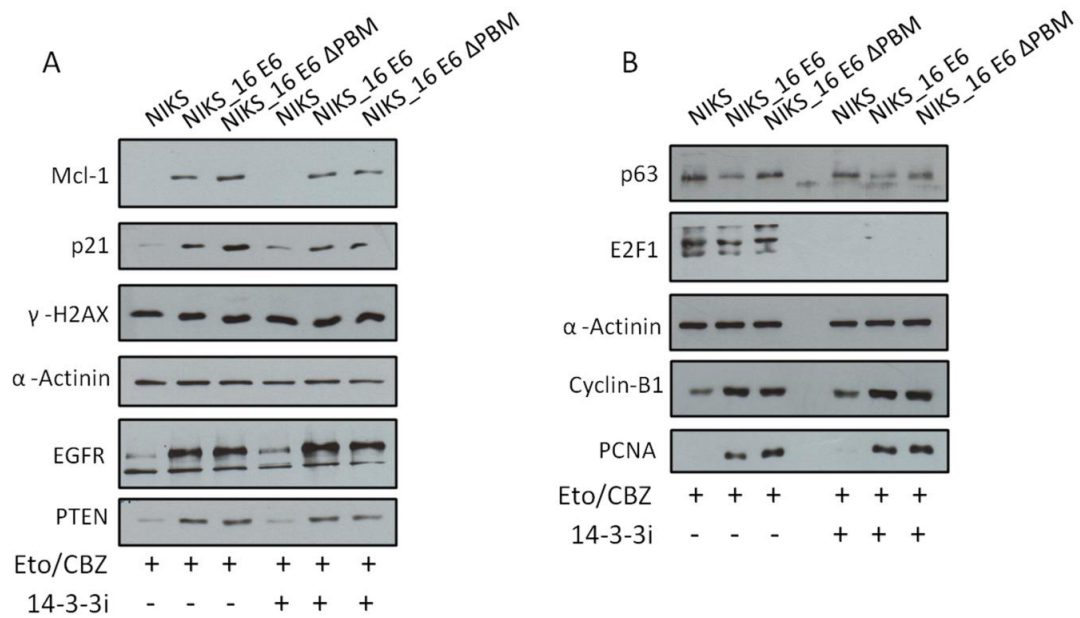


Figure 26. Inhibition of 14-3-3 alters E6 PBM-mediated p53 inactivation with regard to certain genes.

A and B) Western blot analysis of p53 target gene expression: Mcl-1, p21, p63, EGFR, PTEN, Cyclin-B1, PCNA and E2F1 protein levels were examined in NIKS, 16E6 NIKS and 16E6 ΔPBM NIKS upon DNA damage induced by etoposide, together with proteasome inhibitor (CBZ), and in the presence or absence of a 14-3-3 inhibitor. The level of DNA damage was analysed using the DNA damage marker γ-H2AX; α-actinin was used as loading control.

CHAPTER 3: RESULTS

PART:2

Identification of sequence constraints governing Chk1 and PKA phosphorylation of HPV-18 E6

The above studies demonstrate that multiple cellular kinases can phosphorylate E6 in response to a variety of different signaling pathways and stress responses. However, a critical question remains as to how to separate phosphorylation of E6 by different kinases from the basic PDZ binding function conferred by T156 residue. Therefore, I was next interested in analyzing whether different mutations could be introduced into the E6 PBM which would retain PDZ recognition but abolish phosphorylation of E6 by different kinases.

We have previously defined residue R153, just upstream of the HPV-18 E6 PBM, as being essential for PKA recognition of the E6 PBM (Kuhne, Gardiol et al. 2000, Boon and Banks 2013, Boon, Tomaic et al. 2015), but we have no information on whether sequences downstream of the phospho-acceptor site at T156 might also play a role in E6 phosphorylation by Chk1, PKA or AKT. This is important, as we know that non-canonical residues play an important role in E6 PDZ selection (Thomas, Dasgupta et al. 2008, Luck, Charbonnier et al. 2012, Thomas, Myers et al. 2016), and we wanted to determine if this was also true for kinase recognition of E6. In order to investigate this we produced three mutations within the HPV-18 E6 PBM: the wildtype sequence RRETQV was mutated to RRETQstop, RRETstop and RRETAA (Figure 27A). The wild type and mutant E6 proteins were expressed as GST fusion proteins and purified, then subjected to in vitro phosphorylation with purified Chk1, PKA or AKT. First, we examined which upstream residues were required for Chk1 recognition of E6. As shown in Figure 27B, the R153A mutation abolishes Chk1 phosphorylation of E6, as has previously been shown for PKA phosphorylation. Removal of either of the last two carboxy-terminal residues has a marked deleterious impact upon the ability of both Chk1 and PKA to phosphorylate HPV-18 E6, highlighting the critical importance of residues downstream of T156 for efficient kinase recognition (Figure 27B and C). Interestingly, the double mutant, RRETAA shows greatly reduced phosphorylation with Chk1 but not with PKA or

AKT. Differences in kinase recognition were also observed in the case of the RRETQstop mutant, where Chk1 can still phosphorylate the E6 PBM at T156, whereas PKA cannot. However, in the case of AKT phosphorylation, mutations downstream of the phospho-acceptor site had minimal impact on AKT kinase recognition (Figure 27D).

These results demonstrate that both Chk1 and PKA phosphorylation of HPV-18 E6 are critically dependent upon the presence of two amino acids immediately downstream of the phospho-acceptor site, but alanine substitutions are perfectly acceptable, while AKT kinase recognition does not depend on the presence of these residues. To our knowledge, this is the first demonstration that a Chk1 and PKA phospho-acceptor site can be affected by the proximity of the protein's carboxy-terminus.

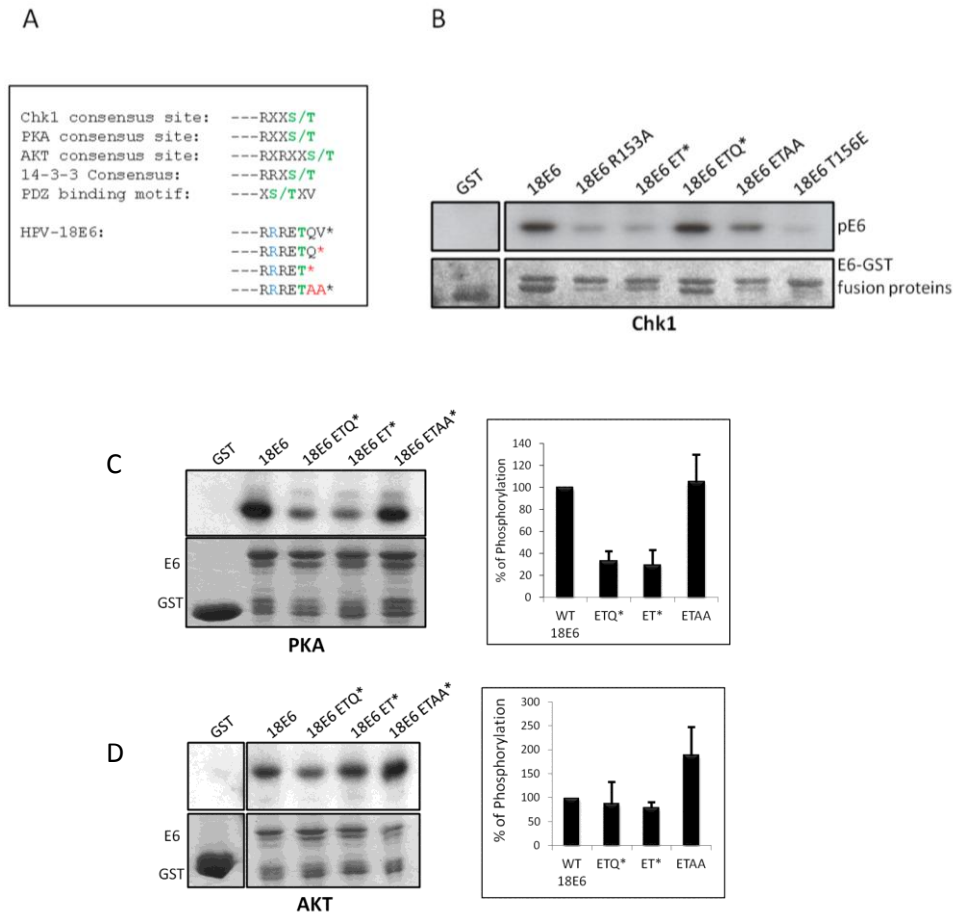
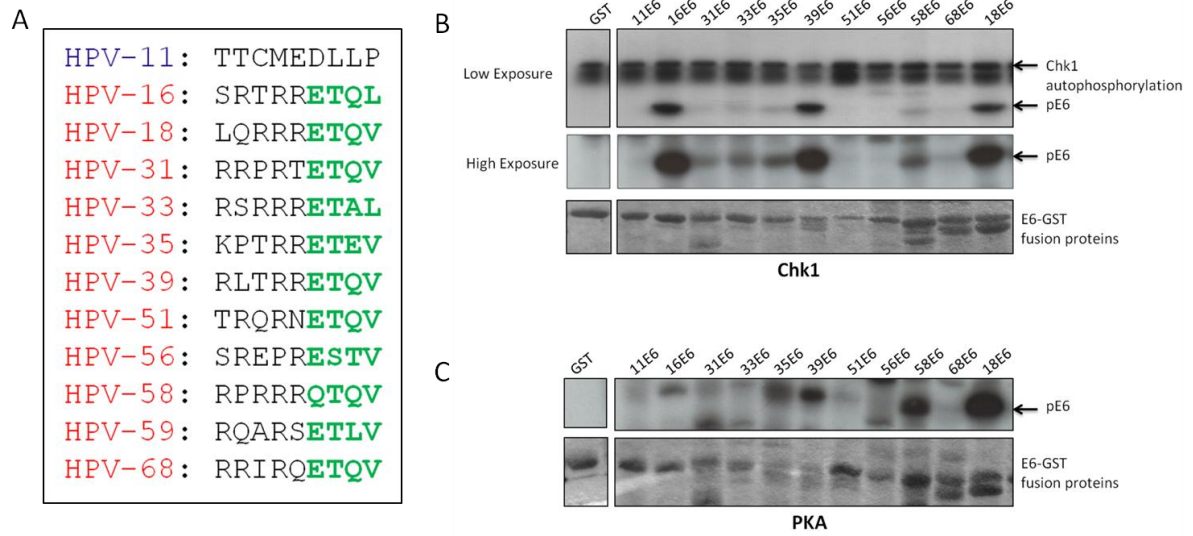


Figure 27. Kinase recognition of HPV-18 E6 is dependent on both upstream and downstream residues of the phospho-acceptor site.

A) The consensus phosphorylation motifs recognized by Chk1, PKA and AKT, plus the 14-3-3 and PDZ interaction motifs are shown. The sequence of the carboxy-terminus of HPV-18 E6 and the various mutations generated within this region are shown below. The purified GST fusion proteins were incubated with either Chk1 (B), PKA (C) or AKT (D) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Proteins were then analysed by SDS-PAGE and autoradiography. The upper panels show representative autoradiogram of the different *in vitro* phosphorylated wild type and mutant 18E6 GST fusion proteins; the lower panels show the Coomassie Blue-stained gel. The histograms show the quantification of autoradiograms from at least three independent experiments; standard deviations are shown.

Different HPV E6 types show different susceptibility to phosphorylation by Chk1

Previous studies had also shown a significant variation in how different HPV E6 types could be phosphorylated by AKT or PKA (Boon and Banks 2013, Boon, Tomaic et al. 2015). Having shown that, both upstream and downstream residues around the phospho-acceptor site within HPV-18 E6 PBM play critical role in kinase recognition, we next wanted to ascertain if this is conserved throughout different HPV E6 types. Although, all high risk HPV E6 possesses conserved PBM at their carboxy-terminus, there is a high degree of variation in their sequences upstream and downstream of phospho-acceptor site, as shown in Figure 28A. To investigate this, we initiated series of *in vitro* kinase assays using purified Chk1 and PKA kinases with different HPV E6 GST-fusion proteins, in the presence of radiolabeled ATP. As can be seen in Figure 28B, HPV-16, -39 and -18 E6 are heavily phosphorylated by Chk1 kinase, whilst HPV- 31, -33, -35, -51, -56 and -68 are weakly phosphorylated. Similar assay was performed using PKA kinase and we observed very striking differences in their phosphorylation, as shown in Figure 28C. The HPV- 18 E6 and -39 E6 are the strongest substrates of PKA, followed by HPV-58, -35 and -16 E6 respectively. As with the Chk1 kinase, HPV- 31, -33, -51, -56 and -68 are weakly phosphorylated by PKA. The high degree of phosphorylation by both Chk1 and PKA correlates with the enriched upstream arginine residues in the case of HPV-16, -18 and -39. The HPV-35 and HPV-58 are strong substrates of PKA but not of Chk1, potentially due to the presence of downstream inhibitory residues. These results suggest that, in agreement with previous studies, the non-canonical residues play critical role in kinase recognition.



Courtesy: Arushi Vats

Figure 28. Different HPV E6 types display different levels of phosphorylation.

A) The carboxy-terminus sequence of different HPV E6 types. B and C) The purified GST fusion proteins were incubated with either Chk1 or PKA and [γ - 32 P]ATP. Proteins were then subjected to SDS-PAGE and autoradiographic analysis. Upper panel represents autoradiogram of different *in vitro* phosphorylated wild type and mutant 18E6 GST fusion proteins; lower panel, the Coomassie blue-stained gel.

Non-canonical residues play an important role in E6-PDZ and E6-14-3-3 recognition

We were next interested in ascertaining how these mutations in the HPV-18 E6 PBM would affect PDZ and 14-3-3 recognition. To do this we first performed an *in vitro* interaction assay using purified HPV-18 E6-GST-fusion proteins with *in vitro*-translated, radiolabeled MAGI-1 and Discs Large (Dlg). The results obtained are shown in Figure 29A and B, and show that mutation or removal of any of the amino acid residues downstream of T156 destroys the ability of E6 to interact with PDZ substrates, confirming that PDZ recognition requires the complete consensus recognition motif (ETQV).

We then monitored the ability of these mutants to interact with 14-3-3 following phosphorylation. The different HPV-18 E6-GST-fusion proteins were purified and subjected to *in vitro* phosphorylation with Chk1 kinase using non-radioactive ATP; following extensive washing, they were incubated with purified 14-3-3 γ and 14-3-3 ζ . The bound protein was detected by western blotting and the results are shown in Figure 30A and B. As expected, the R153A and T156E mutants fail to interact with 14-3-3 proteins, as they cannot be phosphorylated by Chk1. The RRETstop mutation, which abolishes Chk1 phosphorylation, also destroys 14-3-3 γ and 14-3-3 ζ interaction, whilst the RRETQstop mutant interacts with both 14-3-3 γ and 14-3-3 ζ in a manner close to wild type HPV-18 E6, which is consistent with the efficiency with which this mutant is phosphorylated by Chk1. Surprisingly, the RRETAA double mutant, which shows reduced levels of phosphorylation, appears to interact strongly with 14-3-3 ζ , but fails to interact with 14-3-3 γ . These results suggest that different 14-3-3 isoforms might also interact with different E6s in subtly different ways depending on the precise sequence of the PBM as well as the degree to which E6 is phosphorylated.

To support the finding that 14-3-3 proteins can associate directly with HPV-18 E6 in a phosphorylation-dependent manner and to confirm that their interaction is not mediated by E6-bound PKA or Chk1 kinase, we made use of ATP- γ -S in our kinase assays. This form of ATP is nonhydrolyzable, or is poorly hydrolyzed compared with ATP. As can be seen in Figure 31A, 14-3-3 γ can associate with HPV-18 E6 when phosphorylated with PKA, but when we used ATP- γ -S, it fails to interact with E6. Similarly, 14-3-3 ζ interaction with E6 is inhibited in the presence of ATP- γ -S (Figure 31B), suggesting that the 14-3-3 association with E6 is direct and phosphorylation-dependent.

Next, we also wanted to confirm the interaction of PKA with different E6-GST fusion proteins in the presence of ATP- γ -S. As can be seen in Figure 31C, the western blot analysis of catalytic subunit of PKA kinase shows that, the kinase is able to recognize the wild type and all different PBM mutants of E6. This suggests that differences in the phosphorylation of different E6 mutants are not due to kinase recognition of PBM but due to overall requirement of specific amino acids within the motif.

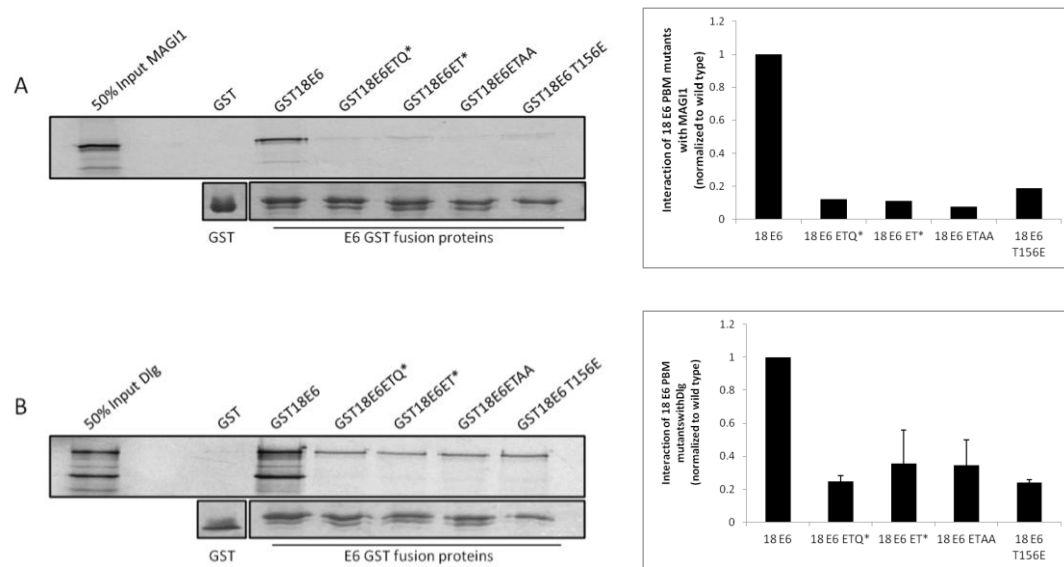


Figure 29. Mutations in HPV-18 E6 PBM region abolish PDZ interactions.

The wild type and mutant 18E6 GST fusion proteins were incubated with *in vitro*-translated radiolabeled ([³⁵S]methionine) MAGI1 (A) or Dlg (B) as indicated. Following extensive washing, bound proteins were detected by using SDS-PAGE and autoradiography. Upper panel, autoradiogram; lower panel, the Coomassie Blue-stained gel. The histograms show quantification of interaction of different 18E6 PBM mutants with MAGI1 (data shown from 2 experiments) or Dlg1 (data shown from 3 experiments) (as indicated), normalized to wild type 18E6.

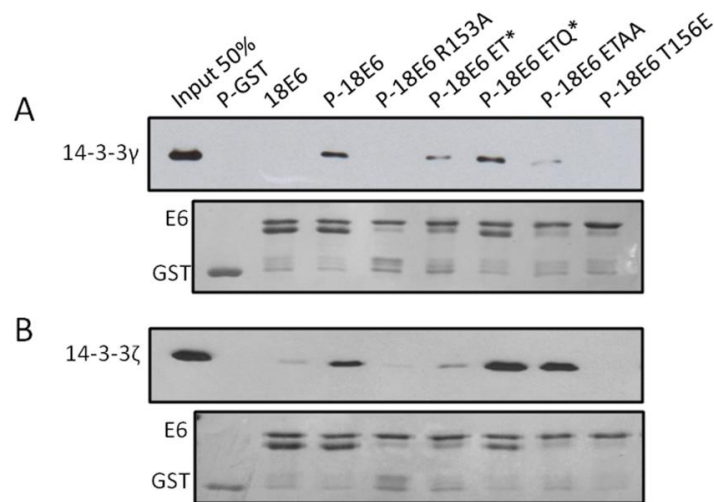


Figure 30. Non-canonical residues in the HPV-18 E6 PBM play an important role in E6-14-3-3 recognition.

A direct interaction assay with GST-E6 fusion proteins and purified 14-3-3 γ (A) and 14-3-3 ζ (B). Purified GST fusion proteins were either untreated or subjected to phosphorylation (indicated as “P”) with Chk1 in the presence of non-radiolabeled ATP. They were then incubated with purified recombinant 14-3-3 γ and 14-3-3 ζ . After extensive washing, the bound protein was detected by Western blotting using anti-14-3-3 γ or 14-3-3 ζ antibody (upper panel). Ponceau Red staining of the nitrocellulose membrane shows the GST fusion proteins (lower panel).

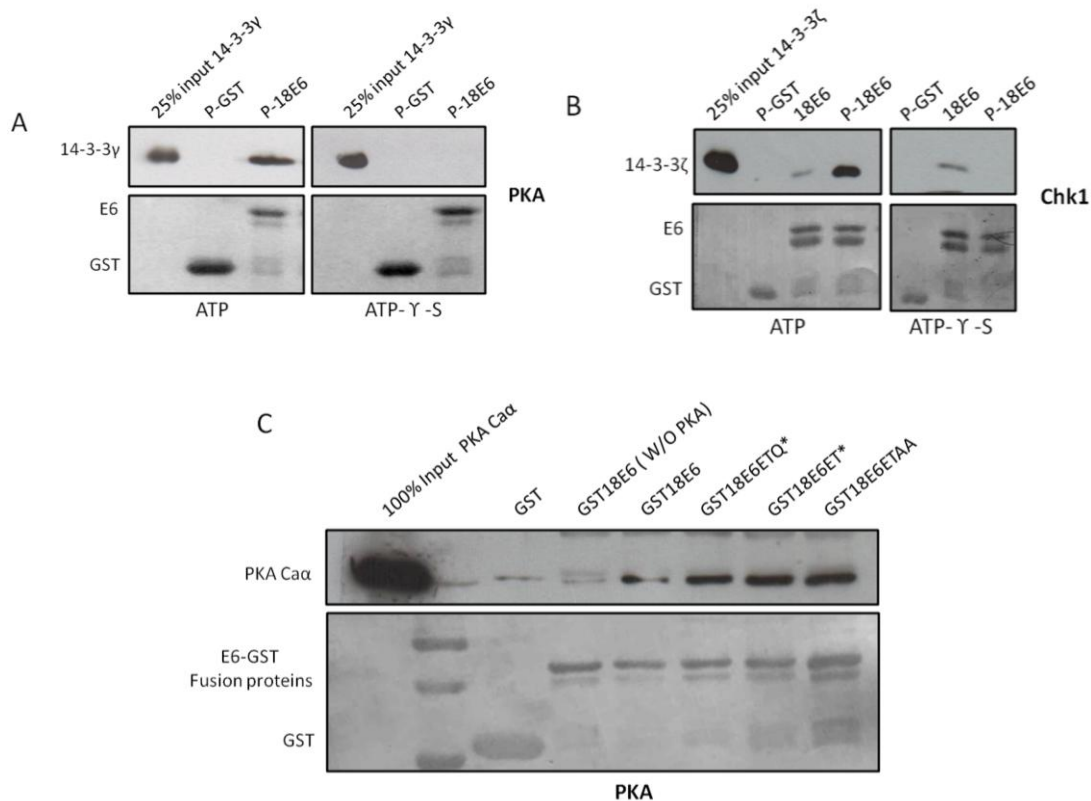


Figure 31. The phospho-dependent interaction of HPV-18 E6 with 14-3-3 is direct.

A direct interaction assay between GST-E6 fusion proteins and purified 14-3-3γ (A) and 14-3-3ζ (B). Purified HPV-18 E6 GST fusion proteins were either untreated or subjected to phosphorylation (indicated as “P”) with PKA (A) or Chk1 (B) in the presence of non-radiolabeled ATP or ATP-γ-S. They were then incubated with purified recombinant 14-3-3γ or 14-3-3ζ. After extensive washing, the bound protein was detected by Western blotting using anti-14-3-3γ or 14-3-3ζ antibody (upper panel). Ponceau Red staining of the nitrocellulose membrane shows the GST fusion proteins (lower panel). C) Western blot analysis of interaction of PKA Cα kinase with different E6-GST fusion proteins in the presence of ATP-γ-S.

Having shown that the different HPV E6 types can be phosphorylated differentially, the next question was: Is there any direct biological consequence of this E6 phosphorylation? As described and demonstrated earlier, HPV-18 E6 phosphorylation within the PBM controls p53 functions by inactivating its transcriptional transactivation ability with respect to certain promoters. We wanted to test whether E6s from the different HPV types could regulate p53 functions in a similar way, and to determine whether it correlates with their capacity to be phosphorylated.

To do that, we used the p53-responsive promoter MDM2 cloned into a luciferase reporter plasmid (MDM2-Luc), also including Renilla luciferase as a transfection efficiency control. The p53-null H1299 cells were transfected with the reporter construct, together with p53 and different HPV E6 expression plasmids, in the presence or absence of CBZ (to rescue the degradation of p53 by E6) and etoposide (in order to induce E6 phosphorylation). After 24hrs cells were harvested and luciferase activity was measured using the dual-luciferase assay system, and the results are shown in Figure 32. As can be seen, the HPV-18 E6 Δ PBM mutant allows a significant increase in p53 transcriptional activity in the presence of proteasome inhibitor and etoposide, whilst this activity is suppressed in the case of wild type HPV-18 E6, suggesting a E6 phosphorylation-dependent p53 inactivation. Interestingly, in the presence of HPV-56 E6 and HPV-68 E6, both of which are poorly phosphorylated, there is a marked increase in the p53 transcriptional activity in the presence of proteasome inhibitor and etoposide. These data suggest that E6 phosphorylation plays an important role in regulating p53 functions.

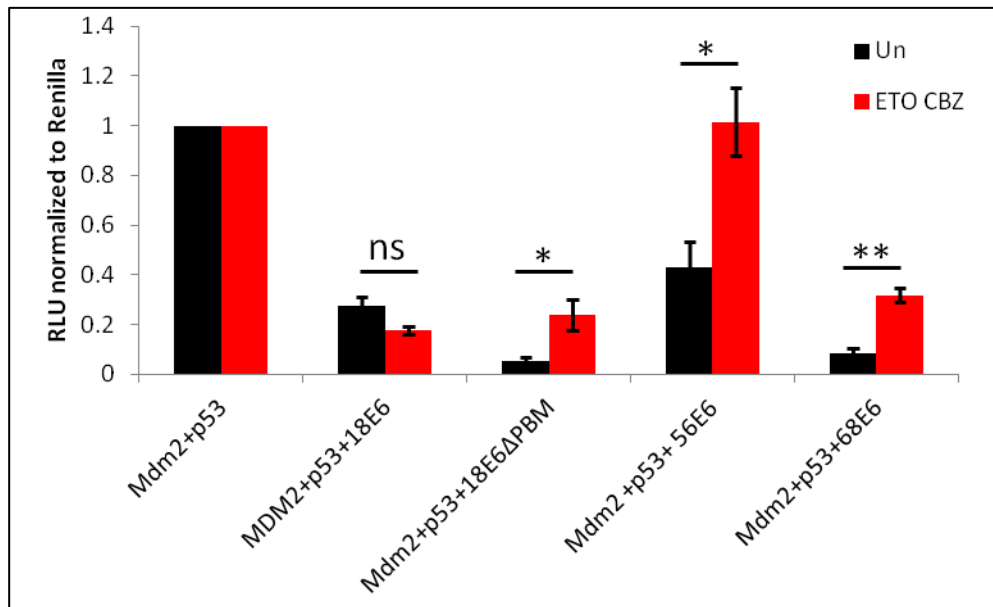


Figure 32. Phosphorylation of the E6 PBM affects the transcriptional transactivation of a p53-responsive promoter.

H1299 cells were transfected with the MDM2-Luc construct (the MDM2 promoter cloned upstream of a luciferase gene), together with plasmids expressing p53 and the following HPV E6 proteins: HPV-18 E6, HPV 18 E6 ΔPBM mutant, HPV- 39 E6, HPV-56 E6 and HPV-68 E6, in the presence or absence of the proteasome inhibitor CBZ. The histograms show the luciferase assay results from at least three independent experiments, quantified using Student's t-test, and showing the standard error of mean. Also shown are the p values (* <0.05, ns=non significant) for the changes in the relative luciferase activity.

CHAPTER 3: RESULTS

PART:3

Investigation of how phosphorylation modulates E6 utilisation of the E6AP ubiquitin ligase

In the preceding sections, I investigated how different signaling pathways could modulate E6 function via phosphorylation of the E6 PBM. Whilst there is also an indication of how post-translational modifications of specific E6 substrates might affect their susceptibility to E6 targeting, there is as yet no information on how this might modulate E6 utilisation of its most important interacting partner, the E6AP ubiquitin ligase. This seems particularly important since recent studies have demonstrated that PKA phosphorylation of E6AP at position T485 resulted in decreased enzymatic activity, whilst a non-phosphorylatable mutant of E6AP appeared to increase the levels of ubiquitin-mediated degradation of its substrates (Yi, Berrios et al. 2015). These studies demonstrated that, an autism-linked missense mutation disrupts the phosphorylation site and this results in the synaptic dysfunction and autism pathogenesis. In order to firstly confirm the identity of the PKA phospho-acceptor site in E6AP, the wild type and T485A mutant (phospho-dead) of E6AP were expressed as GST fusion proteins, purified and then subjected to *in vitro* phosphorylation with the catalytic subunit of PKA in the presence of radiolabeled-ATP. As can be seen in Figure 33A, the phosphorylation of the E6AP T485A mutant is greatly decreased compared to wild type. This result confirms that the T485 residue is a major PKA phospho-acceptor site in E6AP.

In order to begin to understand the role of phospho-regulation of E6AP in the context of HPV E6-induced degradation of its target proteins, we first wanted to generate a stable cell line in which the endogenous E6AP expression had been ablated. This was necessary since previous studies had shown that E6AP forms a multimeric complex with E6 (Martinez-Zapien, Ruiz et al. 2016) and it can also induce its own autoubiquitination and degradation (Kao, Beaudenon et al. 2000). Therefore, it was important that we perform these studies in E6AP null cells. To do this, we performed genome editing of HEK293 cells to knock out the endogenous E6AP using the

CRISPR-Cas9 system. Following selection, single-cell cloning and initial screening by DNA sequencing (Figure 33C), the cells were then further analysed by western blotting to measure any residual levels of E6AP. As can be seen from Figure 33B, clone 1 was completely deficient for full-length E6AP, and sequencing showed that it has a stop codon at residue 430; therefore this clone was chosen for all subsequent analyses.

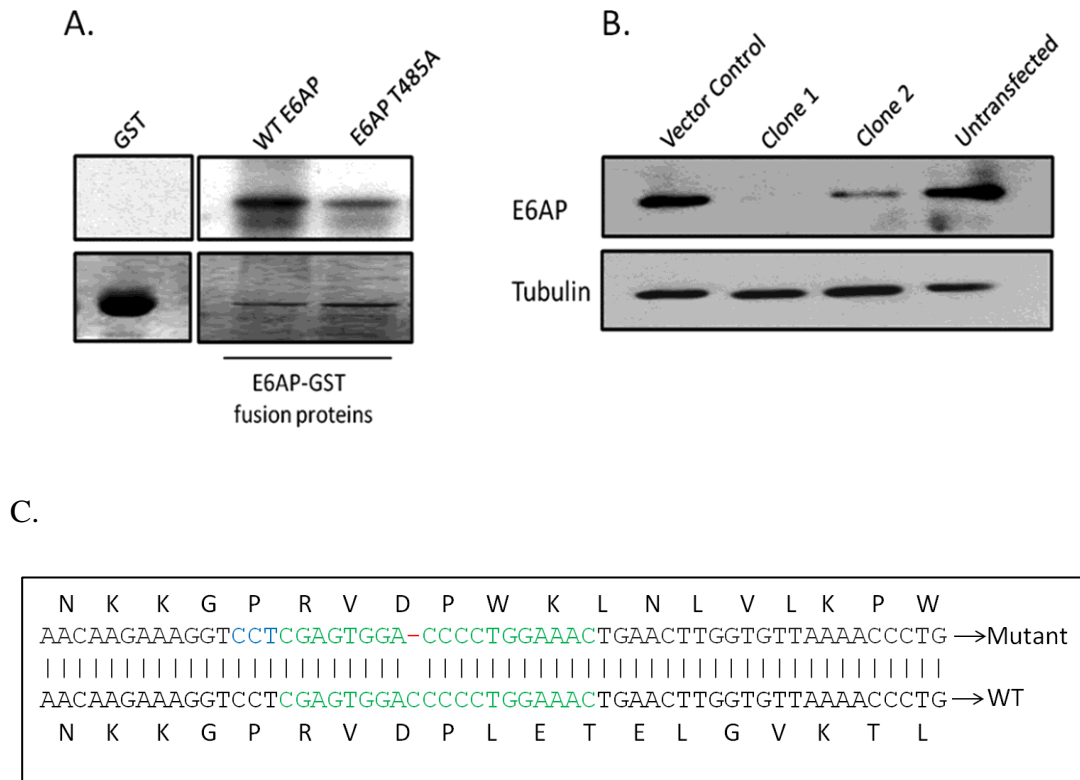


Figure 33. The E6AP T485 residue is the major phospho-acceptor site for PKA.

A) The purified E6AP wild-type and T485A mutant GST fusion proteins were incubated with PKA and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Proteins were then subjected to SDS-PAGE and autoradiographic analysis. Upper panel: autoradiogram of *in vitro* phosphorylated wild type and mutant E6AP-GST fusion proteins; lower panel, the Coomassie Blue-stained gel. B) Western blot analysis of clones obtained from genome-editing E6AP demonstrating the efficiency of gRNAs in targeting E6AP. C) DNA sequencing result showing generation of mutation in the CRISPR/gRNA targeted E6AP locus.

Phosphorylation of E6AP at T485 affects E6AP degradation of Ring1B

Previous studies had analysed the effect of phospho-regulation of E6AP upon the degradation of HHR23A (Yi, Berrios et al. 2015), and we wanted to study this in the E6AP-null background. Therefore, E6AP-null HEK293 cells were transfected with HHR23A, together with plasmids expressing either wild-type E6AP, or the T485A (non-phosphorylatable) or T485E (phospho-mimic) mutant. After 24hrs the cells were harvested and proteins analysed by western blotting. The results are shown in Figure 34A. Despite multiple attempts, we were unable to demonstrate that E6AP was capable of inducing degradation of HHR23A, and the reasons for this are currently unclear. In order to determine whether E6AP could increase the ubiquitination of HHR23A, we transfected the E6AP null HEK293 cells with V5-tagged HHR23A expression plasmid, together with the wild-type and mutant E6AP constructs and HA-tagged ubiquitin expression plasmids. After 24hrs the cells were harvested and immunoprecipitated with anti-HA-conjugated agarose beads. The ubiquitinated HHR23A was then detected by western blotting using the anti-V5 antibody. The results in Figure 34B, show that HHR23A is not polyubiquitinated in the absence of E6AP, but is markedly polyubiquitinated in the presence of E6AP. In agreement with previous studies (Yi, Berrios et al. 2015), there is further ubiquitination of HHR23A in the presence of the E6AP T485A mutant, while the T485E mutant shows slightly lower levels of polyubiquitination of HHR23A. Thus, despite not promoting degradation of HHR23A in our assays, the wild type and T485A mutant of E6AP can promote HHR23A polyubiquitination. Similarly, we tested another group of reported E6AP substrates: PML-1, PML-2, PML-3 and PML-5 (Louria-Hayon, Alsheich-Bartok et al. 2009). E6AP-null HEK293 cells were transfected with Flag-PML, together with the wild type E6AP expression construct. After 24hrs the cells were harvested and proteins analysed by western blotting. As can be seen in Figure 34C, we observed no degradation of PML isoforms in the presence of wild type E6AP, rather there was an apparent increase in PML levels. Taken together, these results suggest that there should be great care in interpreting which cellular proteins are normal degradation substrates of E6AP.

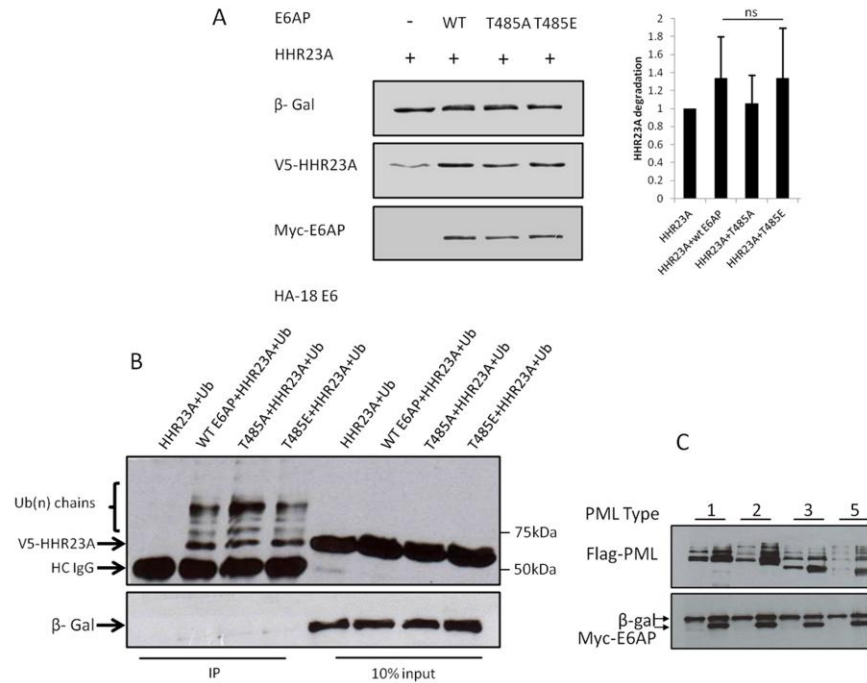


Figure 34. Regulation of E6AP through T485 phosphorylation does not affect the degradation of E6AP's normal substrates HHR23A and PML.

A) E6AP-null HEK293 cells were transfected with the indicated plasmids and after 24hrs the cells were harvested and protein levels analysed by western blotting. β - galactosidase acted as a control for transfection efficiency. The right hand panel shows the relative values of HHR23A degradation from at least 3 independent assays. Standard errors of means are shown. (P value: ns>0.05). B) Cells were transfected with the indicated expression plasmids, and also treated with proteasome inhibitor. After 24hrs cells were harvested and subjected to immunoprecipitation with anti-HA-conjugated agarose beads. The polyubiquitinated HHR23A was then detected by western blotting with anti-V5 antibody. The right hand side of each blot shows the input levels of HHR23A used in the immunoprecipitations. C) E6AP-null HEK293 cells were transfected with the different isoforms of PML plasmids with or without wild type E6AP, after 24hrs the cells were harvested and protein levels analysed by western blotting. β -galactosidase acted as a control for transfection efficiency.

We then analysed another reported substrate of E6AP, Ring1B, which has also been shown in previous studies to be degraded by E6AP (Mortensen, Schneider et al. 2015) (Zaaroor-Regev, de Bie et al. 2010). The E6AP-null HEK293 cells were transfected with plasmids expressing Ring1B, together with wild type E6AP, the T485A phospho-destroyed mutant or the T485E phospho-mimic mutant. After 24hrs the cells were harvested and proteins analysed by western blotting. The results in Figure 35A demonstrate that E6AP alone can induce the degradation of Ring1B, and this is further increased in the presence of the T485A mutant, which supports previous studies indicating an increased degradation capability with the T485A mutant (Yi, Berrios et al. 2015). Surprisingly, the phospho-mimic T485E mutant consistently retained some degradation activity in these assays. In order to determine whether E6AP could increase the ubiquitination of Ring1B, we transfected the E6AP-null HEK293 cells with FLAG-tagged Ring1B expression plasmid, together with the different E6AP mutants and HA-tagged ubiquitin expression plasmids. Cells were treated with proteasome inhibitor, CBZ. After 24hrs the cells were harvested and immunoprecipitated with anti-FLAG conjugated agarose beads. The ubiquitinated Ring1B was then detected by western blotting using the anti-HA-HRP antibody. The results in Figure 35B, show that there is a weak polyubiquitination of Ring1B in the absence of E6AP, but that this increases dramatically in the presence of wild type E6AP. There is a further slight increase in ubiquitination of Ring1B in the presence of the E6AP T485A mutant, whilst the T485E mutant only weakly ubiquitinates Ring1B. Taken together, these results, using a different substrate of E6AP, largely support previous studies, which indicate that mutation of the T485 phospho-acceptor site can have an effect upon E6AP's enzymatic activity.

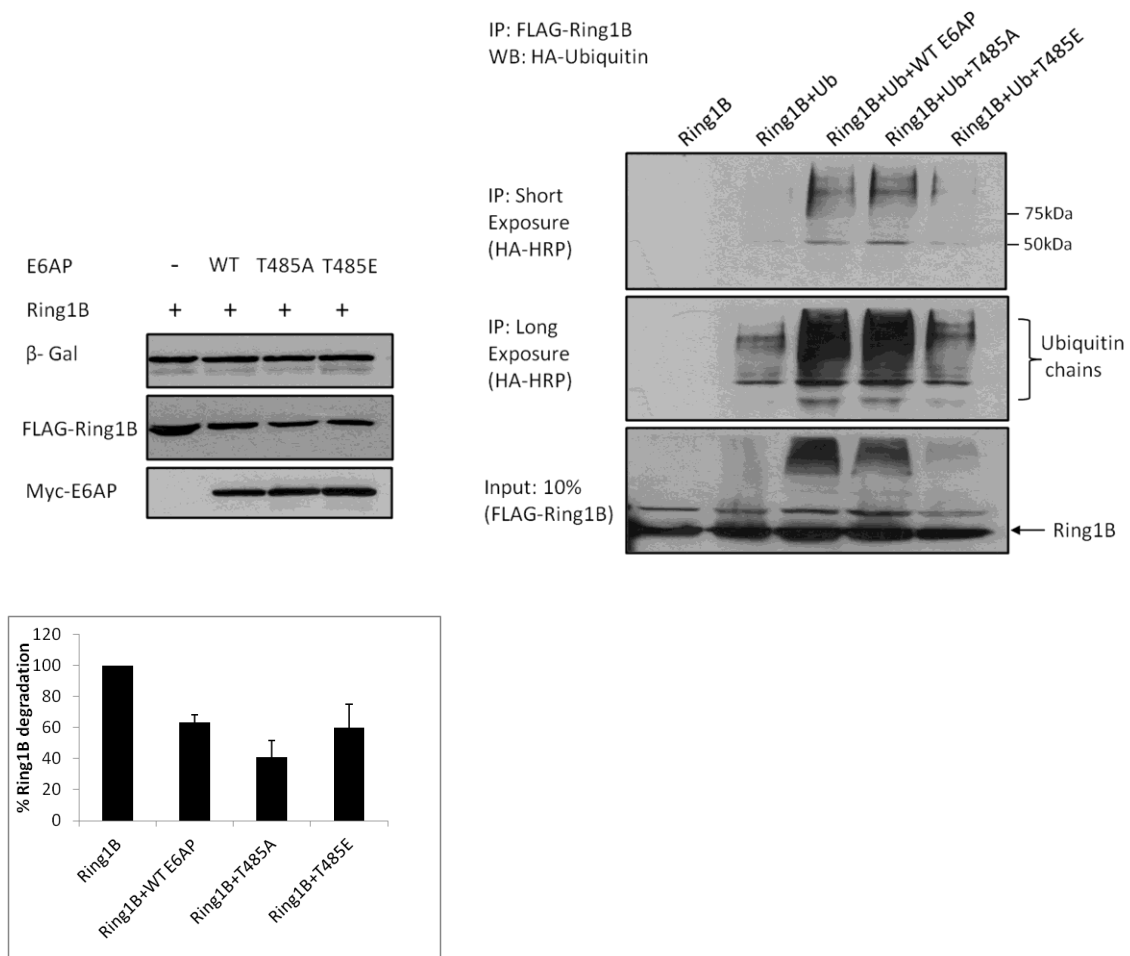


Figure 35. Regulation of E6AP through T485 affects the degradation of E6AP's normal substrate, Ring1B.

A) E6AP-null HEK293 cells were transfected with the indicated plasmids and after 24hrs the cells were harvested and protein levels analysed by western blotting. β-galactosidase acted as a control for transfection efficiency. B) Cells were transfected with the indicated expression plasmids, and treated with proteasome inhibitor, after 24hrs were harvested and subjected to immunoprecipitation with anti-FLAG-conjugated agarose beads. The polyubiquitinated Ring1B was then detected by western blotting with anti-HA-HRP antibody. The lower panel shows the input levels of Ring1B used in the immunoprecipitation. The histogram shows percentage Ring1B degradation in the presence of wild type and mutants of E6AP.

HPV E6 overrides the normal phospho-control of E6AP at T485.

Considering that E6AP is recruited by E6 for many of its activities, we were next interested in determining whether the phospho-mimic T485E or phospho-destroying T485A amino acid substitutions in E6AP could affect the ability of HPV E6 to direct the degradation of p53. To do this, the E6AP-null HEK 293 cells were co-transfected with either HPV-16 E6, HPV-18 E6 or HPV-31 E6, together with p53 and the wild type or mutant E6AP expression plasmids. After 24hrs the cells were harvested and the protein levels analysed by western blotting. The results in Figure 36A, B and C demonstrate that p53 is not degraded by E6 if E6AP is absent, and this is in agreement with many other previously published studies (Scheffner, Huibregtse et al. 1993). Co-transfection of wild type E6AP with E6 promotes p53 degradation, and there is a concomitant increase in the levels of E6 expression, which is also consistent with previous observations showing that E6AP is required for maintaining E6 stability (Tomaic, Pim et al. 2009). Most interestingly, however, when we co-transfect either the T485A or the T485E mutants, p53 appears to be degraded with an efficiency similar to that observed with the wild type E6AP. These results suggest that phosphorylation of T485 has little effect upon the ability of either HPV-16, HPV-18 E6 or HPV-31 E6 to utilize E6AP for the degradation of p53.

We then proceeded to investigate whether another HPV E6 substrate was similarly unaffected by T485 phospho-regulation. To do this we analysed Dlg, which is a PDZ domain-containing substrate of HPV E6 (Gardioli, Kuhne et al. 1999). The E6AP-null HEK293 cells were transfected with a Dlg expression plasmid, together with the different E6AP expression constructs and HPV-16 E6. After 24hrs the cells were harvested and the protein levels analysed by western blotting. The results in Figure 36D demonstrate that Dlg degradation by E6 is also unaffected by either the T485A or T485E amino acid substitutions.

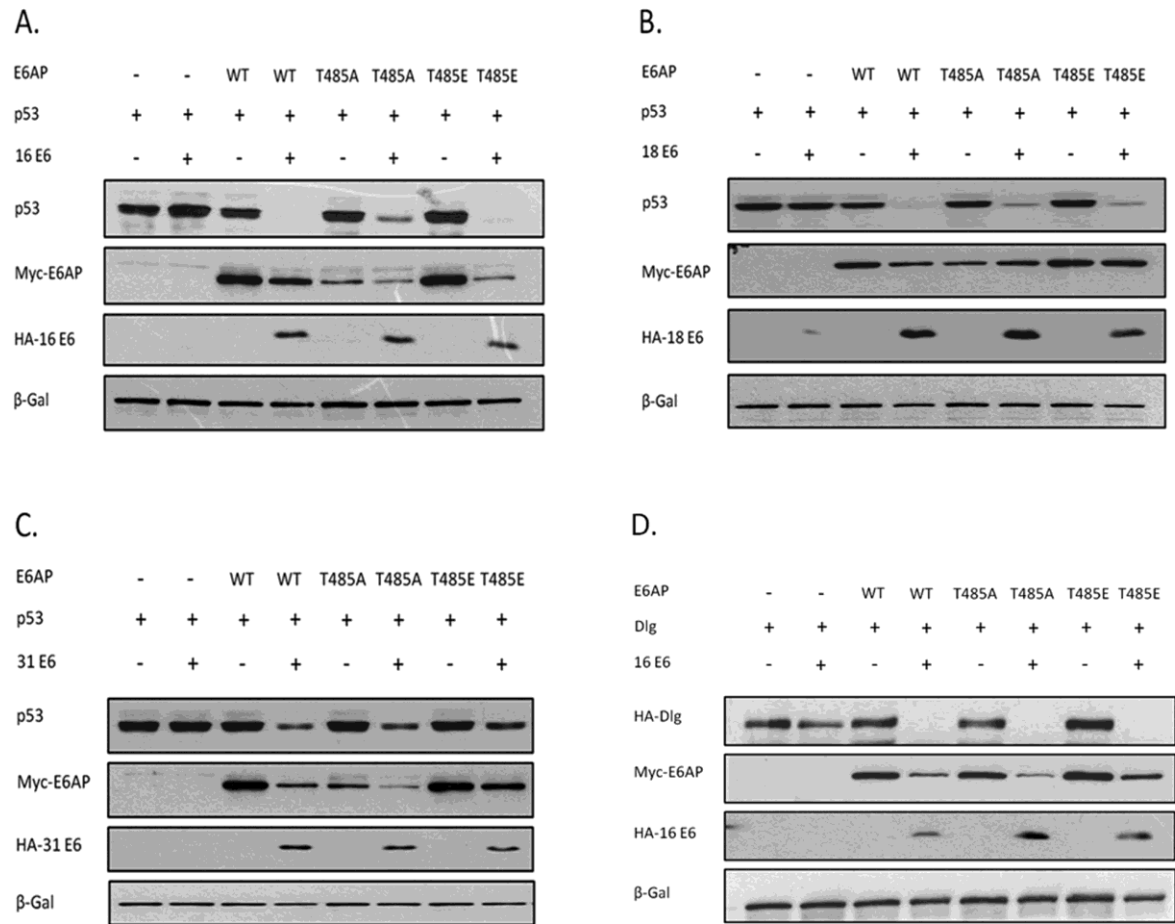


Figure 36. E6AP regulation at T485 has no effect on HPV E6 degradation of p53 and Dlg. E6AP-null HEK293 cells were transfected with plasmids expressing E6AP and p53, as indicated, plus plasmids expressing either HPV-16 E6 (Panel A), HPV-18 E6 (Panel B) or HPV-31 E6 (Panel C). After 24hrs the cells were harvested and protein levels analysed by western blotting. β -galactosidase acted as a control for transfection efficiency in all assays. D) E6AP-null HEK293 cells were transfected with E6AP, Dlg-1 and HPV-16 E6 and after 24hrs the cells were harvested and protein levels analysed by western blotting. β -galactosidase acted as a control for transfection efficiency. N=3.

We also tested the autodegradation of wild type E6AP and the T485A and T485E mutants. To do that we transfected the different E6AP expression plasmids into E6AP-null H1299 cells, and after 24 hrs we performed half-life experiments using a cycloheximide pulse-chase for different time points. As can be seen in Figure 37A, we observed that wild-type E6AP and the T485A mutant show equivalent rates of self-turnover during the period of the assay, whilst the autodegradation ability of T485E mutant is markedly reduced, and these results are consistent with previously reported results (Yi, Berrios et al. 2015).

It was clear from the analysis of p53 degradation in Figure 36 that there are significant differences in how the E6AP mutants are degraded by the different HPV E6 proteins, with HPV-16 E6 and HPV-31 E6 targeting the wild type and the two mutants with similar efficiency, whilst HPV-18 E6 appears to be incapable of degrading the T485A and T485E mutants. In order to confirm this, the assay was repeated without the presence of exogenously added substrate and the results obtained are shown in Figure 37B and C, where it can be seen that HPV-16 E6 effectively degrades wild type E6AP and the T485A and T485E mutants, whilst HPV-18 E6 is largely defective in degrading the T485A and T485E mutants. These results suggest that the autodegradation activity of the T485E mutant can be re-activated by HPV-16 E6 and HPV-31 E6, but not by HPV-18 E6. In contrast, the T485A mutant, which has intrinsically more autoubiquitination activity (Yi, Berrios et al. 2015), can be further degraded by HPV-16 E6 and HPV-31 E6, but not by HPV-18 E6, indicating intriguing differences in how these viral oncoproteins redirect E6AP activity.

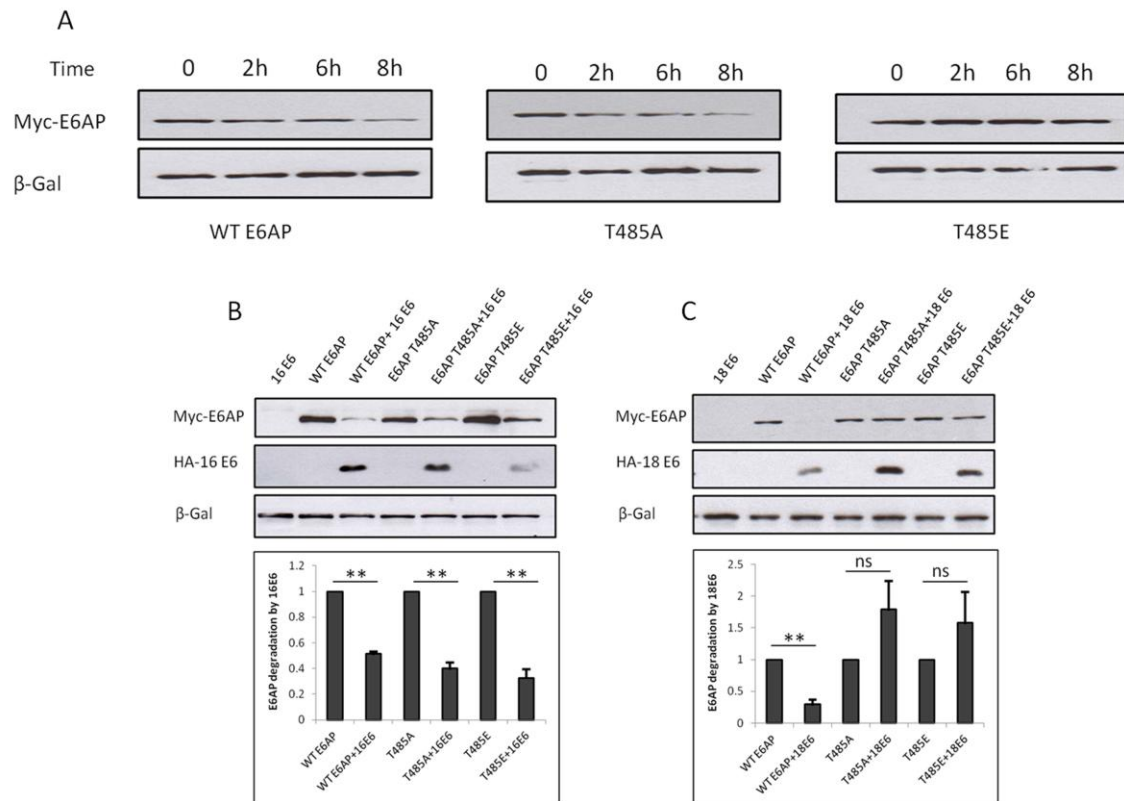


Figure 37. Different HPV E6s uncouple normal PKA phospho-regulation of E6AP.

A) E6AP-null HEK293 cells were transfected with plasmids expressing E6AP and 24hrs post transfection, the cells were treated with Cycloheximide for indicated time points and then the cells were harvested and protein levels analysed by western blotting. E6AP-null HEK293 cells were transfected with plasmids expressing E6AP, plus plasmids expressing either HPV-16 E6 (Panel B) or HPV-18 E6 (Panel C). After 24hrs the cells were harvested and protein levels analysed by western blotting. β -galactosidase acted as a control for transfection efficiency in all assays. The bottom panels show the statistical quantification using Student's t-test. Values are shown are means from at least 3 independent experiments; standard error of the mean is shown. ** $P < 0.005$.

HPV E6 recruits phospho-E6AP to the nucleus in a 14-3-3-dependent manner.

Having found that HPV E6 can redirect E6AP activity independently of its T485 phosphorylation status, we were interested in investigating how the subcellular distribution of phosphorylated and non-phosphorylated forms of E6AP might appear in cells derived from a cervical cancer, and whether E6 might have any impact upon the subcellular distribution of these different forms of E6AP. In order to do this, we performed a series of immunofluorescence analyses in HeLa cells, which contain HPV-18 E6. The cells were transfected with siRNA E6/E7 to determine whether the viral oncoproteins might modulate the distribution of the different forms of E6AP. At the same time, siRNA E6AP was also transfected to verify the specificity of the anti-E6AP antibodies. As can be seen in Figure 38A, control HeLa cells have very low levels of p53 and E6AP. There are also very low levels of phospho-E6AP, although interestingly, there does appear to be some variability in the staining for phospho-E6AP, suggesting there might be an element of cell cycle control in its phosphorylation. Most interestingly, when HeLa cells are transfected with siRNA against E6/E7 (Figure 38B), there is, as expected, a dramatic increase in the levels of nuclear p53 and E6AP, whilst the phospho-E6AP levels increase, primarily within the cytoplasmic compartment. These results suggest that phospho-E6AP normally resides within the cytoplasm, whilst non-phosphorylated E6AP is mostly found with the nucleus. However, in the presence of E6, both forms of E6AP accumulate within the nucleus.

In order to verify the E6-mediated nuclear accumulation of phospho-E6AP we repeated the immunofluorescence analysis in the presence of the proteasome inhibitor MG132. The results obtained are shown in Figure 38A (lower panel) and, as can be seen, proteasome inhibition results in a dramatic increase in the amount of both total and phosphorylated E6AP within the nucleus.

Differential subcellular localization of phosphorylated proteins is often mediated through the activity of the 14-3-3 family of proteins (Muslin and Xing 2000). Since E6 can also interact with certain 14-3-3 isoforms (18), we were initially interested in investigating whether phosphorylation of E6AP at T485 could confer interaction with 14-3-3 proteins. To do this, we purified GST.E6AP wild type and GST.E6AP T485A fusion proteins and subjected them to in vitro phosphorylation with purified PKA in the absence of radiolabel, and then performed binding assays with recombinant 14-3-3 γ . As a positive control we also included HPV-18 E6 in the assays. The results in Figure 39 show that, whilst there is a strong increase in the ability of phospho-E6 to interact with 14-3-3 γ , which is in agreement with previous results (Boon and Banks 2013), phosphorylation of E6AP only results in a very modest increase in interaction with 14-3-3 γ , although this is dependent upon the presence of an intact phospho-acceptor site at T485. Whether this is a reflection of low levels of phosphorylation of E6AP, or intrinsically weak interaction with this particular 14-3-3 isoform remains to be determined.

In order to investigate a potential role for 14-3-3 in modulating the pattern of E6AP expression, we performed another series of immunofluorescence analyses in HeLa cells, but in this case, we transfected the cells with a plasmid expressing Difoepin, which has been shown previously to block endogenous 14-3-3 proteins from interacting with their substrates (Xu, Fulop et al. 2010) (Cao, Yang et al. 2010). As can be seen in Figure 40A, there is a significant increase in the amount of phospho-E6AP in the cytoplasm following transfection of Difoepin. In contrast, Difoepin does not alter the sub-cellular distribution of phospho-E6AP when E6 is also removed (Figure 40B), where the majority of phosphorylated E6AP is found in the cytoplasm. Taken together, these results indicate that nuclear accumulation of phospho-E6AP in the presence of HPV-18 E6 is in part dependent upon 14-3-3.

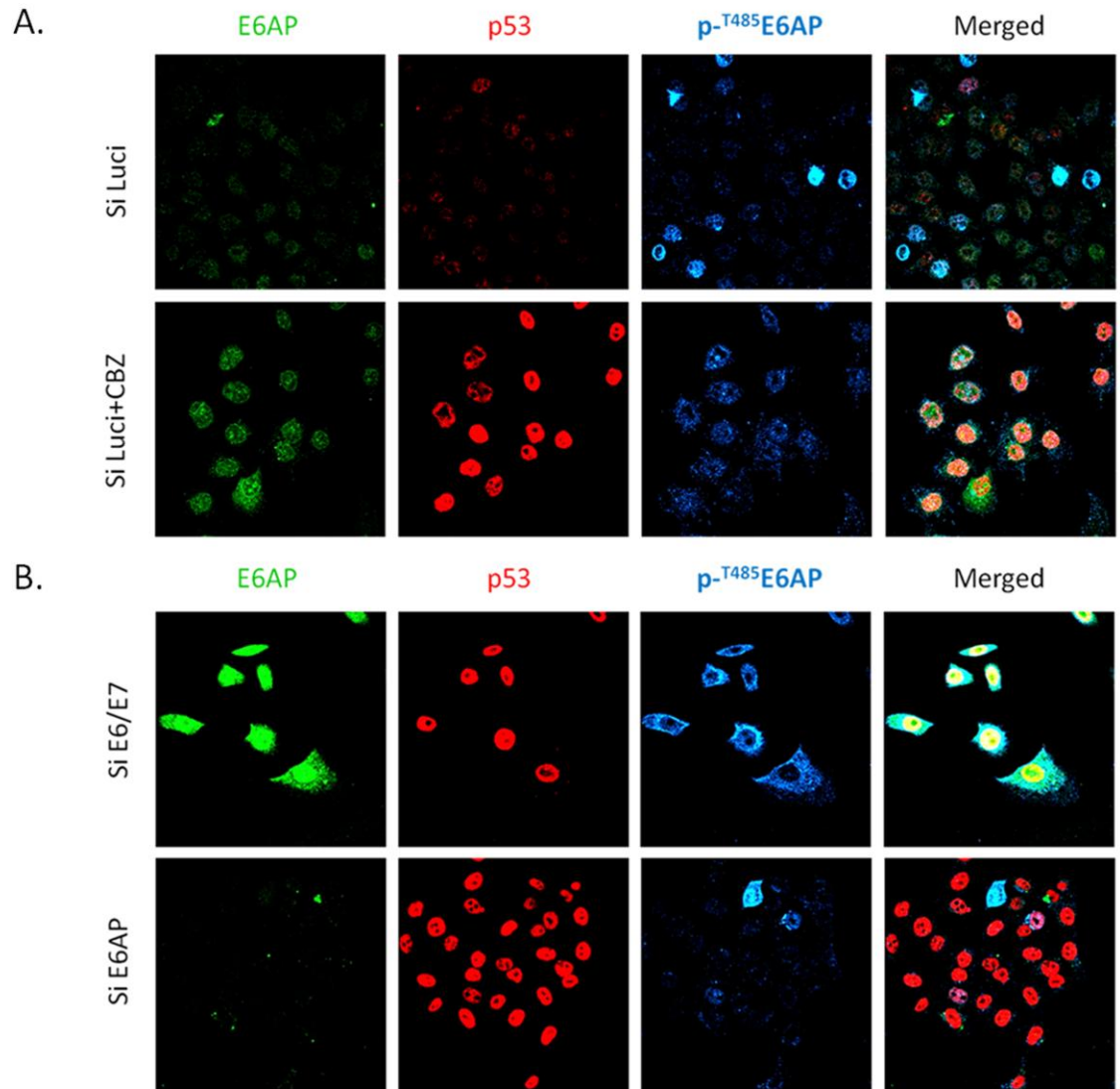


Figure 38. Phospho-forms of E6AP have distinct subcellular distribution in HeLa cells.

A) HeLa cells were transfected with control siRNA against luciferase and after 72hrs were incubated for a further 3hrs with either DMSO or the proteasome inhibitor CBZ. The cells were then fixed and patterns of protein expression monitored using anti-E6AP, anti-p53 and anti-phosphoT485 E6AP antibodies respectively. B) HeLa cells were transfected with either siRNA E6/E7 or siRNA E6AP and after 72hrs the cells were fixed and stained for E6AP, p53 and phosphoT485 E6AP. N=3

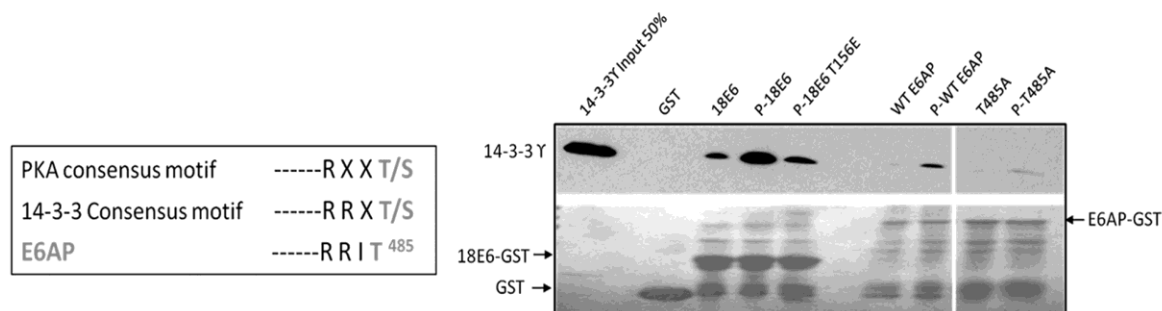


Figure 39. Phosphorylation of E6AP at T485 confers its interaction with 14-3-3γ.

The left panel shows the PKA and 14-3-3 phospho-consensus motifs. The right panel shows a direct interaction assay with purified 14-3-3γ. Purified GST fusion proteins were either untreated or subjected to phosphorylation (indicated as “P”) with PKA in the presence of non-radiolabeled ATP. They were then incubated with purified recombinant 14-3-3γ. After extensive washing, the bound protein was detected by Western blotting using anti-14-3-3γ antibody (upper panel), Ponceau staining of the nitrocellulose membrane is shown in the bottom panel.

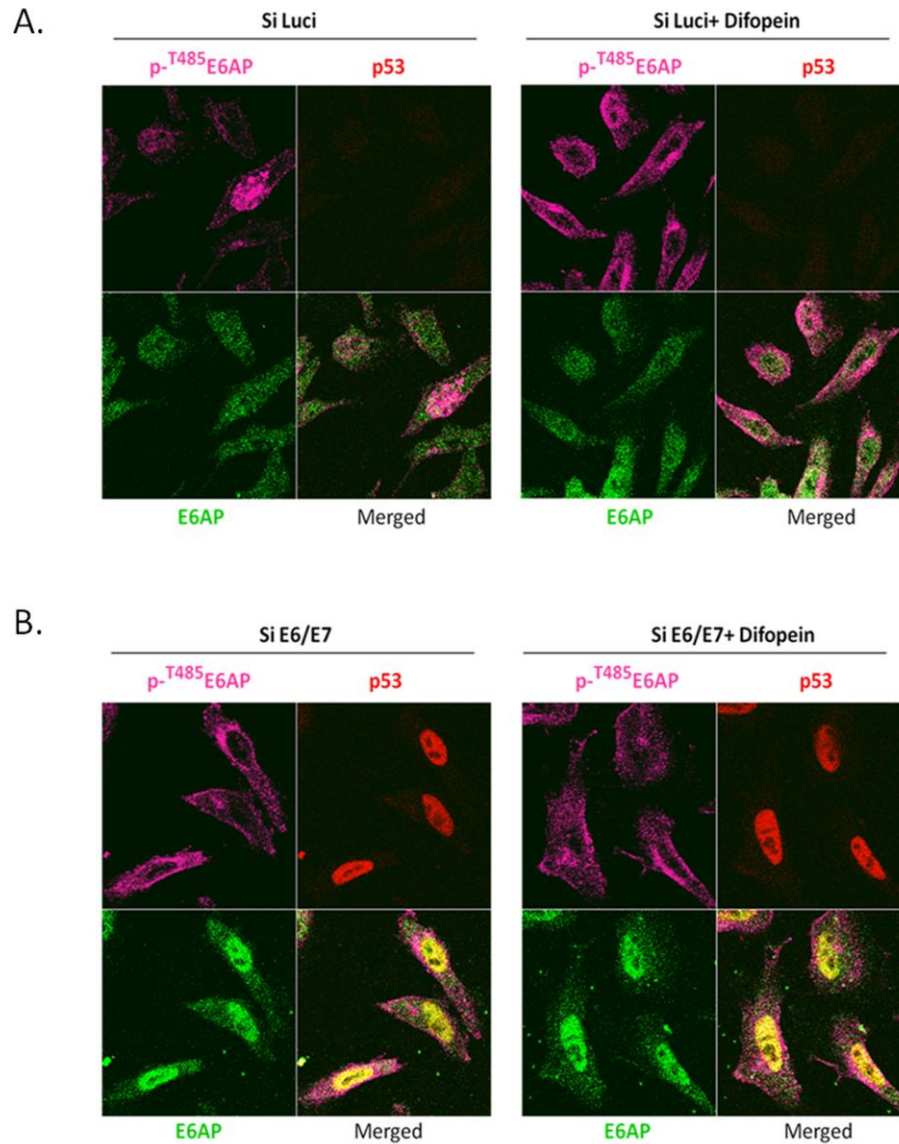


Figure 40. HPV E6 recruits phospho-E6AP to the nucleus in a 14-3-3 dependent manner.

A) HeLa cells were transfected with control siRNA against luciferase, plus Difopein in the right panel, after 72hrs the cells were fixed and patterns of protein expression monitored using anti anti-phosphoT485 E6AP, anti-p53 and anti-E6AP antibodies respectively. B) HeLa cells were transfected with either siRNA E6/E7 (left panels) or with siRNAE6/E7 plus with Difopein (right panels), after 72hrs the cells were fixed and stained for anti-phosphoT485 E6AP, anti-p53 and anti-E6AP antibodies respectively.

CHAPTER 3: RESULTS

PART:4

HPV-31 E6 displays a complex pattern of phosphorylation regulation

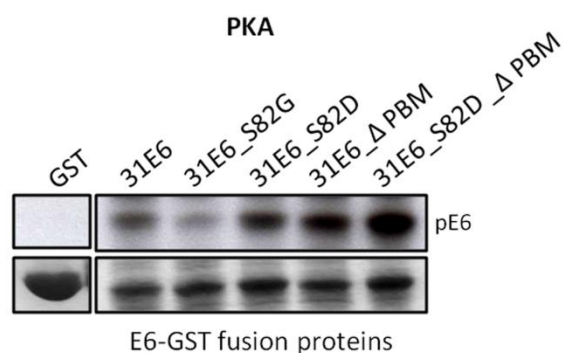
Previously we have shown that there are interesting differences in how different kinases recognize E6 oncoproteins from different HPV types. We also know from previous studies that, unlike HPV-18 E6, HPV-31 E6 has a particularly complex pattern of phospho-regulation. Thus, HPV-31 E6 appears to be phosphorylated by AKT within the PBM, but the PKA phospho-acceptor site probably resides at S82 (Boon, Tomaic et al. 2015). To verify that HPV-31 E6 is phosphorylated by PKA at S82, I made S82A, S82G and S82D substitutions, with the A and G substitutions acting to destroy the phospho-acceptor site, whilst the D substitution acts as a phospho-mimic, as shown in Figure 41A. The proteins were expressed as GST fusion proteins, purified and subjected to *in vitro* phosphorylation with PKA. The results shown in Figure 41B demonstrate that the S82G mutation greatly reduce phosphorylation by PKA, thus confirming S82 as the major phospho-acceptor site for PKA on HPV-31 E6. Intriguingly, the S82D mutant appeared to retain wild type levels of phosphorylation, suggesting that the incorporation of a negative charge at this position can promote phosphorylation by PKA at an additional site within the HPV-31 E6 protein. In addition, generation of the double mutation i.e. S82D/ Δ PBM also resulted in high levels of HPV-31 E6 phosphorylation. This indicates that PKA can phosphorylate HPV-31 E6 at two different sites, one at S82 and other as yet unidentified site which is recognized following phosphorylation at S82.

I also performed phosphorylation assays using AKT and, as can be seen from Figure 41C, the phosphorylation is only slightly decreased in the three substitution mutants at S82, but is abolished in the double mutant, S82A/ Δ PBM, confirming that the major phospho-acceptor site for AKT resides within the HPV-31 E6 PBM.

A

	82	PBM
HPV-31E6:	-----YRYSVY-----	RRPRTE T QV
HPV-31E6 S82A:	-----YRYAVY-----	RRPRTE T QV
HPV-31E6 S82G:	-----YRYGVY-----	RRPRTE T QV
HPV-31E6 S82D:	-----YRYDVY-----	RRPRTE T QV
HPV-31E6 S82A-ΔPBM:	-----YRYAVY-----	RRPRT*
HPV-31E6 S82D-ΔPBM:	-----YRYDVY-----	RRPRT*

B



C

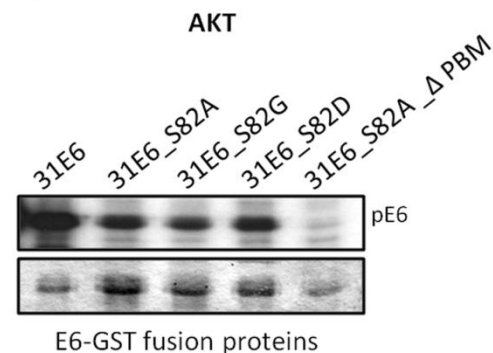


Figure 41. The major PKA phospho-acceptor site in HPV-31 E6 resides at S82 residue.

A) Schematic representation of different mutants generated in HPV-31 E6 GST fusion proteins. B) and C) The purified HPV-31 E6 GST fusion proteins were incubated with PKA or AKT and [γ - 32 P] ATP (indicated as “P”). Proteins were then subjected to SDS-PAGE and autoradiographic analysis. Upper panels represent autoradiograms of different *in vitro* phosphorylated wild type and mutant HPV-31 E6 GST fusion proteins; lower panels show the Coomassie Blue-stained gels.

The HPV-31 E6 phospho-acceptor site at S82 controls p53 degradation

Recent structural studies on HPV-16 E6 suggest that the region around S82 in HPV-31 E6 is likely to be important for the overall conformation of the E6 oncoprotein (Nomine, Masson et al. 2006). Major functions of E6 at this site include the ability to interact with the E6AP ubiquitin ligase and target p53 for degradation. I was therefore interested in determining whether mutation of HPV-31 E6 at this site might have any impact upon its ability to degrade p53. To investigate this, I performed a series of *in vivo* degradation assays with the S82A and S82G phospho-dead mutants and the S82D phospho-mimic mutant. These were co-transfected with p53 into p53-null H1299 cells, and the levels of p53 expression were analysed by western blotting. The results obtained are shown in Figure 42A and demonstrate that the S82D mutation abolishes the ability of HPV-31 E6 to degrade p53. In contrast, S82A and S82G retain almost wild-type levels of activity. These results demonstrate that alterations at the S82 phospho-acceptor site can impact negatively upon the ability of HPV-31 E6 to target p53 for degradation, suggesting that PKA phosphorylation of HPV-31 E6 could affect the ability of 31E6 to induce the degradation of p53.

To examine how the S82 residue might contribute to HPV31-E6 function, I proceeded to investigate the stability of HPV-31 E6 when phosphorylated at S82. To do this, the H1299 cells were treated with proteasome inhibitor, CBZ, after co-transfection with p53 and either wild-type or mutant HPV-31 E6. The results are shown in Figure 42B and surprisingly, we observed that the HPV-31 E6 S82D phospho-mimic mutant is unstable, thus most likely accounts for the inability of this mutant to degrade p53. Interestingly, when the equivalent mutation was made in HPV-18 E6, there was no major effect upon its ability to degrade p53, again highlighting major differences in the structure/function of these different HPV E6 oncoproteins.

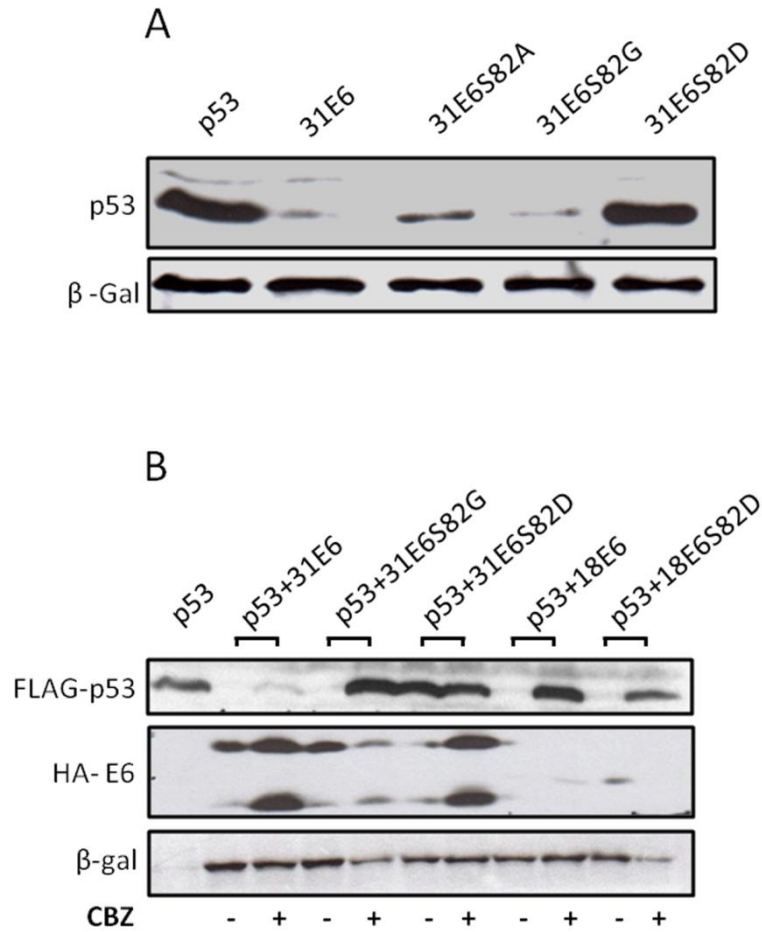


Figure 42. The HPV-31 E6 phospho-acceptor site at S82 controls p53 degradation.

A) *In vivo* assay of the p53-degradation activity of wild type and mutant HPV-31E6. Plasmids expressing p53 and either wild-type or mutant HPV-31E6 were transfected into p53-null H1299 cells. After 24h the protein extracts were subjected to SDS-PAGE and western blotting. The upper panel shows the degradation of FLAG-p53 induced by wild-type or mutant HPV-31 E6, detected by anti-p53 antibody. The lower panel shows the transfection efficiency control, LacZ, detected by anti-β-galactosidase antibody. B) The *in vivo* degradation assay was repeated in the presence and absence of CBZ. Wild-type HPV-18 E6 and the HPV-18 E6S82D mutant were included for comparison.

I was further interested in confirming this result by immunofluorescence. To do this, HA-tagged wild-type 31E6 and the three substitution mutants were co-transfected with p53 into expressed in H1299 cells. After 24hrs the cells were fixed and stained, and the localisation patterns of HPV-31 E6 and p53 were ascertained by immunofluorescence. The results obtained are shown in Figure 43 and demonstrate that wild-type HPV-31 E6 and the S82A and S82G substitution mutants were expressed similarly within H1299 cells, and in no case was any co-expression with p53 observed, suggesting efficient degradation of p53. In contrast, the S82D phospho-mimic, although expressed at lower levels in agreement with the western blotting data, was also found frequently co-localizing with p53. This indicates that the phosphor-mimic at S82 site does not simply induce instability of E6, but rather it is also interestingly weaker in targeting p53 for degradation.

In order to gain more insight into the underlying mechanism, we performed a series of *in vitro* interaction assays with p53 and the HPV-31 E6 mutants. The results obtained are shown in Figure 44 and demonstrate that there are minimal changes in the ability of the mutant and wild type HPV-31 E6 proteins to interact with p53 (Figure 44B). However, there is a striking decrease in the ability of S82D mutant to interact with E6AP.

These results suggest that PKA phosphorylation of HPV-31 E6 at S82 could negatively regulate its ability to degrade p53. This appears to be mediated through an inhibition of interaction with E6AP, which has two direct consequences: reduction of E6 stability and subsequent loss of p53 degradation ability.

Taken together, these results demonstrate a complex pattern of phospho-regulation of HPV-31 E6. I have confirmed the existence of at least two phospho-acceptor sites, one for AKT within the PBM, and one for PKA at the S82 residue. However, the S82D phospho-mimic mutation suggests the existence of a further phospho-acceptor site within the HPV-31 E6 protein.

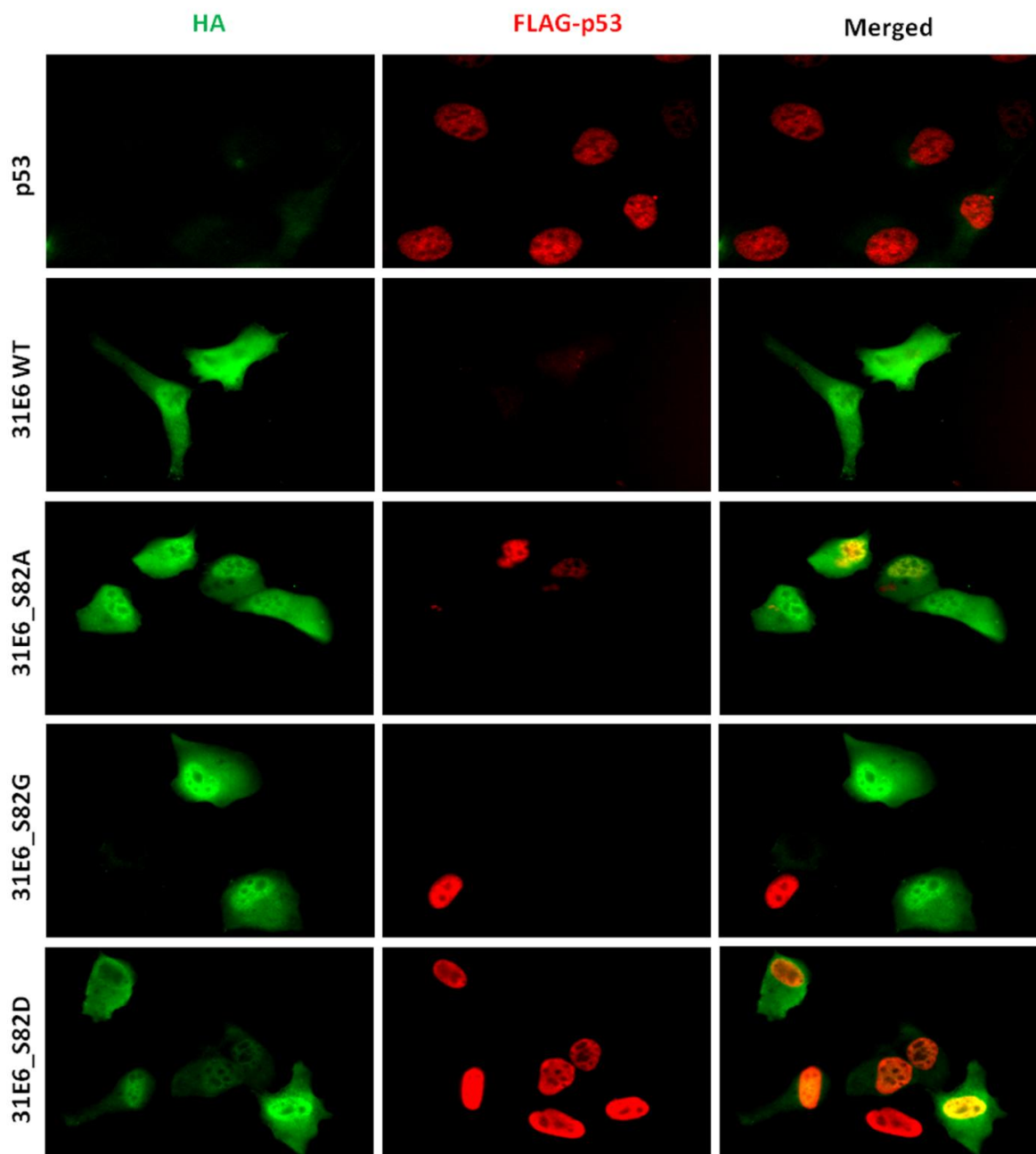


Figure 43. The HPV-31 E6 phospho-acceptor site at S82 controls p53 degradation.

The p53-null H1299 cells were transfected with HA-tagged wild-type 31E6 and mutants of HPV-31 E6 together with p53. The cells were fixed 24h post-transfection and localization of proteins was observed with immunofluorescence (Green indicates HA 31E6 and Red indicated p53).

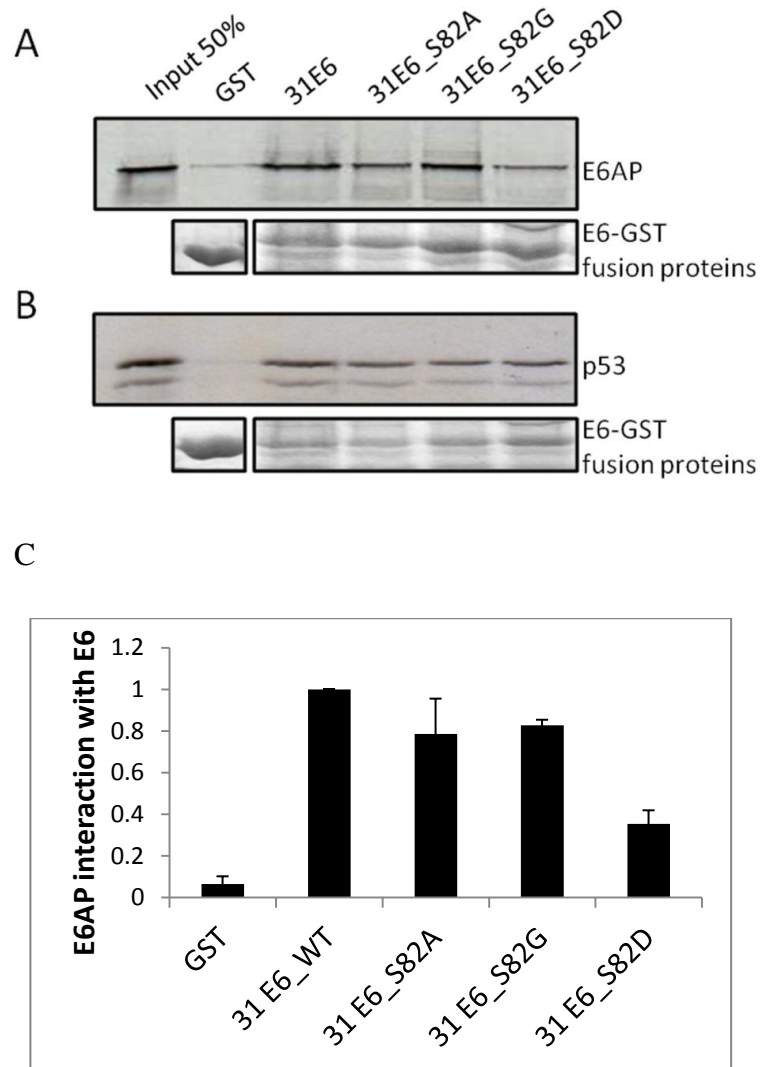


Figure 44. HPV-31 E6 phospho-mimetic mutant show reduced E6AP interaction.

The wild-type and mutant HPV-31 E6 GST fusion proteins were incubated with *in vitro*-translated radiolabeled E6AP (A) and p53 (B), as indicated. Following extensive washing, bound proteins were detected using SDS-PAGE and autoradiography. Upper panel, autoradiogram; lower panel, the Coomassie Blue-stained gel. C) The histogram shows quantification of E6AP interaction with wild type and different mutants of 31E6. The numbers were normalized to wild type control.

CHAPTER 4: DISCUSSION

PART:1

1. Phosphorylation of HPV E6 within the PDZ Binding Motif by DNA Damage Response kinases is linked to E6 inhibition of p53 transcriptional activity

1.1 E6 phosphorylation: A result of stress response

The high-risk HPV E6 oncoproteins are characterized by the presence of a carboxy terminal PBM, embedded within which is a phospho-acceptor site, phosphorylation of which inhibits PDZ binding activity and confers interaction with 14-3-3 proteins (Boon and Banks 2013, Boon, Tomaic et al. 2015). Thus, this site offers the potential of fine-tuning E6's activity with respect to a number of different interaction partners, both within the normal viral life cycle and during the development of malignancy. Previously, we had shown that AKT and PKA could both potentially phosphorylate E6 within the PBM *in vitro* (Boon and Banks 2013, Boon, Tomaic et al. 2015), but we had little evidence about which kinases normally phosphorylate endogenously-expressed E6 in cells derived from cervical cancers. Here we show that E6 is normally only very weakly phosphorylated, if at all, during the normal cell cycle. However, following the induction of a variety of stress response kinases, and in particular those associated with the DDR, there is a dramatic increase in the levels of E6 phosphorylation. The bulk of this phosphorylation appears to be mediated via Chk1, and this therefore directly links regulation of the E6 PBM activity to induction of the DDR, which is known to play a critical role in the normal viral life cycle and potentially in the development of HPV-induced malignancy.

Whilst HPV-16 and HPV-18 E6 had previously been shown to be good substrates for phosphorylation by both AKT and PKA, I first wanted to ascertain when endogenously expressed E6 would be phosphorylated in cells derived from a cervical cancer. We therefore first proceeded to monitor HPV-18 E6

phosphorylation in HeLa cells using two different approaches: double thymidine block and release to follow changes through the cell cycle, and nocodazole treatment to analyse levels of E6 phosphorylation in G2/M. To our surprise we found that thymidine block and release resulted in weak E6 phosphorylation in G1/S arrested cells, whilst nocodazole treatment induced high levels of phosphorylation of E6 in G2/M arrested cells. Since no phosphorylation was observed when the cells cycled normally through G2/M and back into G1 after release from a double thymidine block, this suggested that phosphorylation of E6 might be more linked to a stress response than to normal progression through cell cycle. In addition, in previous unpublished investigations we had been unable to block the nocodazole-induced phosphorylation of E6 using PKA inhibitors (Boon SS thesis), and we therefore reasoned that another pathway of E6 phosphorylation might be operational. Indeed, nocodazole has been implicated in the induction of oxidative stress responses and also in induction of a DDR (Dalton, Nandan et al. 2007, Orth, Loewer et al. 2012, Signoretto, Honisch et al. 2016). In agreement with this, we found that induction of an oxidative stress response by either H₂O₂ or nocodazole was responsible for the very high levels of E6 phosphorylation that were observed following exposure to these agents. Intriguingly, cycloheximide also induces very high levels of E6 phosphorylation, but this appears to be mediated by a different stress response pathway. Since protein synthesis inhibition has been shown to activate several kinases (Zinck, Cahill et al. 1995, Oksvold, Pedersen et al. 2012, Dai, Shi et al. 2013), it is possible that E6 can be phosphorylated by a variety of other stress response kinases. This result also argues for the use of caution in using cycloheximide as a means of monitoring E6 stability over time, as currently we have no information on what this phosphorylation might do to E6 stability. However, previous studies have suggested a bi-phasic half-life for E6, and this could well be linked to changes in E6 phosphorylation status during the course of these assays (Grossman, Mora et al. 1989, Kranjec, Tomaic et al. 2016).

1.2 Involvement of DDR kinases in inducing E6 phosphorylation

Having found that oxidative stress responses were responsible for inducing E6 phosphorylation, we next investigated which DDR pathways might also be involved. We noticed a very high degree of homology within the E6 PBM to a Chk1 consensus recognition site and, indeed, using *in vitro* assays we found that E6 was a very good substrate for phosphorylation by Chk1 at residue T156 within the E6 PBM. In contrast, the closely related Chk2 kinase appeared to be incapable of phosphorylating HPV-18 E6 *in vitro*, further demonstrating the specificity of these assays. *In vivo* the situation was rather more complicated, with different kinases being responsible for E6 phosphorylation, depending upon the specific stimulus. Thus, both H₂O₂- and nocodazole-induced phosphorylation of E6 involves oxidative stress signaling, as we demonstrated by blocking this with NAC, but the kinases involved are different. In the case of nocodazole, Chk1 inhibition clearly blocked E6 phosphorylation, strongly supporting the *in vitro* kinase assays. However, for H₂O₂, Chk2 appeared to be involved, despite the fact that it appeared incapable of phosphorylating E6 *in vitro*. Previous studies have shown that the DDR pathway and Chk2 can also activate PKA (Bensimon, Aebersold et al. 2011, Marazita, Ogara et al. 2012), which we know from our earlier results could also be responsible for phosphorylating E6. Indeed, this does seem to be the case, where H₂O₂-induced phosphorylation of E6 is blocked by treatment with either a Chk2 inhibitor or the PKA inhibitor H89, suggesting that PKA is the kinase responsible for phosphorylating E6 in response to an H₂O₂-induced oxidative stress-induced DDR. Additionally, we observed that the increased phosphorylation of E6 following the inhibition of ATM or Chk1 is mediated largely by PKA. This is in agreement with previous studies suggesting that Chk1 inhibition leads to activation of the ATR pathway (Gagou, Zuazua-Villar et al. 2010, Petermann, Orta et al. 2010, Choi, Toledo et al. 2011) and to further ATR-mediated Chk2 activation in response to H₂O₂ treatment (Zhang, Gao et al. 2014), which subsequently activates PKA

(Bensimon, Aebersold et al. 2011). A model of how these pathways can lead to E6 phosphorylation are shown in Figure 45.

We then extended these studies to investigate whether clinically-relevant inducers of a DDR might also induce E6 phosphorylation. To do this we assessed cisplatin, as an agent capable of intercalating DNA and blocking DNA replication, plus inhibitors of topoisomerase, ribonucleotide reductase and PARP. Interestingly, cisplatin treatment, and also blocking topoisomerase or ribonucleotide reductase all induced a high level of E6 phosphorylation in HeLa cells, which in the case of cisplatin treatment appeared to be largely mediated by Chk1. In contrast, blocking PARP, which has also been linked to induction of a DDR, had no discernible effect upon the levels of E6 phosphorylation, suggesting that the pathways required for phosphorylation of E6 are not activated by PARP inhibition in HeLa cells. This seemed to be linked to the low level of DDR in HeLa cells upon PARP inhibition and suggest that the patterns of E6 phosphorylation might well vary in primary or recently immortalised cells from that seen in HeLa cells, which are fully transformed and have been maintained in tissue culture for many years. Further studies will be required to investigate these aspects in more detail. .

1.3 Biological consequences of E6 phosphorylation

When E6 is phosphorylated in the PBM, this blocks its PDZ recognition and instead confers the ability to interact with 14-3-3 family members. Several previous studies have shown a critical requirement for the activation of DDR pathways in the HPV life cycle, with ATM/ATR in particular playing essential roles in genome amplification (Moody and Laimins 2009, Reinson, Toots et al. 2013, Spriggs and Laimins 2017). Recent studies have also indicated a potential link between the E6 PBM and genome maintenance, with inactivation of p53 appearing to be able to compensate for loss of PBM function (Lorenz, Rivera Cardona et al. 2013). This, therefore, raised the obvious question of whether the E6 PBM might have an

additional function linking it to inactivation of p53. To investigate this, we analysed a panel of different p53-responsive promoters and asked whether an intact PBM could contribute towards the ability of E6 to block p53 transcriptional activity, both in the presence and absence of proteasome inhibition. We find a quite complex picture, where certain promoters, such as that of BAX, are inhibited by E6 regardless of the presence of an intact PBM. In contrast, in the case of Mdm2 and PUMA promoters, the E6 PBM appears to play an essential role in inhibiting p53 transcriptional activity under conditions of proteasome inhibition. Furthermore, using the HPV-18 E6 R153G mutant, which has been shown previously to be defective for phosphorylation but which retains PDZ binding activity (Boon, Tomaic et al. 2015), we found that this was also defective in its ability to block p53 transcriptional activity on the Mdm2 promoter in the presence of proteasome inhibitors. Whilst proteasome inhibition could result in the formation of aberrant complexes consisting of p53, E6AP and E6, which in turn could affect p53 transcriptional activity (Thomas, Massimi et al. 1996), the lack of activity observed using the R153G mutant argues against this, and instead strongly suggests that the major role of the PBM in this scenario is retention of the ability to be phosphorylated. These studies allow us to propose a model, shown in Figure 45, in which a variety of different cellular stresses can induce the phosphorylation of E6 within the PBM, either directly by Chk1 or indirectly through PKA, which in turn can directly contribute to the ability of E6 to block p53 transcriptional activation of a subset of p53 responsive genes. When we analysed various other p53-responsive genes, interacting partners, and regulators by using a p53 RT PCR array, there appeared to be interesting differences in how these genes are regulated in a PBM/phosphorylation-dependent manner. Moreover, some of the tested target genes showed a 14-3-3 dependency for E6-mediated p53 inactivation, as shown by the inhibition of protein expression in the presence of the 14-3-3 inhibitor Dipofein. Obviously, whether there is involvement of cofactors other than 14-3-3 is, as yet, undetermined and the precise mechanisms of E6 PBM inhibition of p53 transcriptional activity via 14-3-3, requires further investigation.

In summary, the above studies provide compelling evidence of a direct link from the induction of oxidative stress, to the induction of a DDR, to the phospho-regulation of cancer-causing HPV E6 oncoproteins, and to their ability to perturb p53 transcriptional activity on a subset of p53-responsive promoters. This is the first example of PBM-PDZ recognition being regulated by DDR kinases, and begins to explain how HPV can make use of the ATM/ATR pathway that is induced during viral infection, whilst at the same time overcoming some of the deleterious effects that would also be expected. These results are also interesting in a broader context of the potential regulation of PBM-PDZ recognition in response to DDR signaling, not only in HPV, but also in regulating different cellular processes, and in particular those linked to the regulation of cell polarity. For example, the interacting partner of Dlg, Net1 (Garcia-Mata, Dubash et al. 2007) and the Scrib interacting partner Vangl1 (Anastas, Biechele et al. 2012) all have PBMs that have phospho-acceptor sites that are remarkably similar to a Chk1 consensus. Studies to investigate whether cell polarity might also be subject to regulation through DDR signaling pathways is currently completely unexplored, but these studies suggest that this would certainly be worthy of further investigation.

These results also raise the intriguing possibility that certain HPV types may be more responsive to certain chemotherapeutic strategies, depending upon the specific HPV type and the sequence of the E6 PBM. Future studies will aim to further clarify these possibilities by generating the R153G mutation in the C4-1 cervical cancer cell line using a genome editing approach. It would also be of great interest to study the phosphoregulation of E6 during the viral life cycle. It is tempting to speculate that E6 is presumably phosphorylated during the viral amplification phase, as studies have reported the requirement for the activation of DDR signaling, with ATM/ATR in particular playing essential roles in viral genome amplification in differentiating epithelium. Whether phosphorylation of E6 also plays a role in the progression to malignancy also remains to be determined.

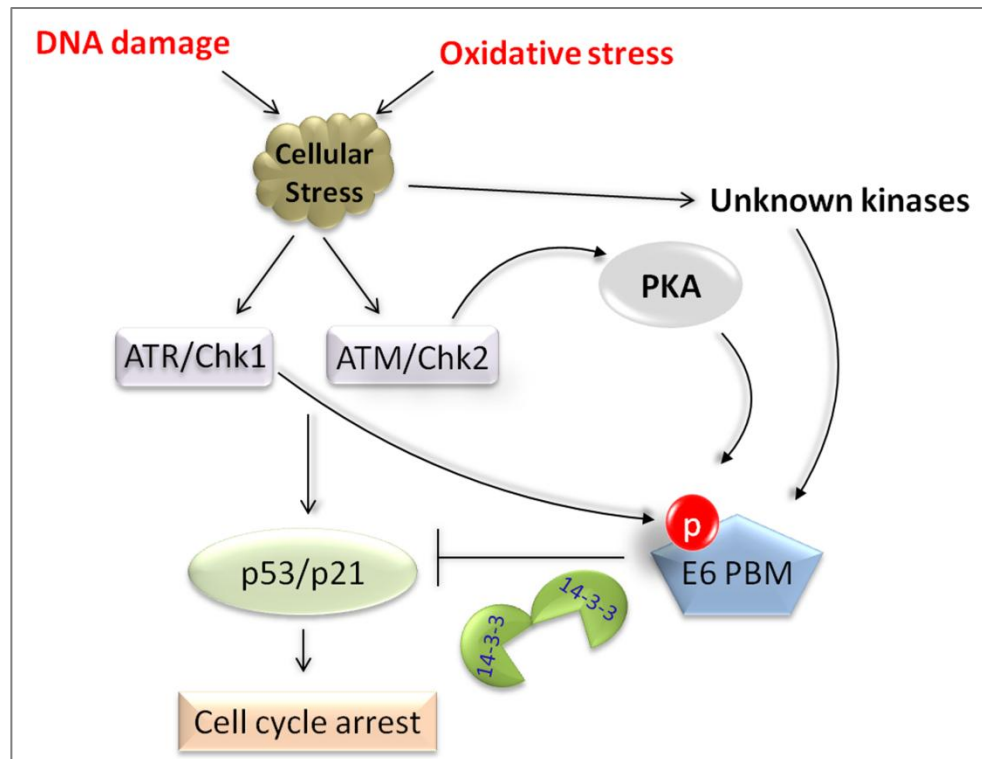


Figure 45. Phosphorylation of the HPV E6 PBM links cellular stress-response signaling to E6 inhibition of p53 transcriptional Activity.

The model summarizes the effect of E6 PBM phosphorylation under various stress conditions. Our results demonstrate the involvement of two major axes: The DNA damage response pathway and other cellular stress response pathway(s) having a number of unknown kinases. Chk1 activation links directly to E6 phosphorylation, whilst Chk2 activation further signals to PKA, which subsequently phosphorylates E6. Our studies provide compelling evidence of a direct link from the induction of oxidative stress and induction of DDR to the phospho-regulation of HPV E6 oncoproteins, potentially interacting with 14-3-3 proteins, to perturb p53's transcriptional activity on a subset of p53 responsive promoters.

PART:2

2. The differential phosphorylation of different HPV E6 types suggests diverse modes of E6 PBM functions

2.1 The non-canonical residues around the E6 PBM play an essential role in E6 phospho-regulation

The above studies demonstrated the regulation of E6 phosphorylation by multiple cellular kinases in response to a variety of stress responses, including DDR signaling. However, it is critical to dissect the two different functions of the E6 PBM and separate its phosphorylation by different kinases from the basic PBM-PDZ interaction. We have previously defined the R153 residue, which resides just upstream of the HPV-18 E6 PBM, as being essential for PKA recognition of the E6 PBM (Kuhne, Gardiol et al. 2000, Boon and Banks 2013, Boon, Tomaic et al. 2015), but we had no information on whether sequences downstream of the phospho-acceptor site at T156 might also play a role in E6 phosphorylation by Chk1, PKA or AKT. This is important, as we know that non-canonical residues play an important role in E6's selection of PDZ-containing targets (Thomas, Dasgupta et al. 2008, Luck, Charbonnier et al. 2012, Thomas, Myers et al. 2016), and we wanted to determine whether this is also true for kinase recognition of E6, which could potentially dissect the dual functionality of the E6 PBM.

In this study we have identified the sequence constraints governing kinase recognition of the E6 PBM. Intriguingly, we find that Chk1 and PKA kinases require the presence of residues downstream of the phospho-acceptor site in HPV-18 E6, with interesting differences in kinase recognition. The PKA recognition appears to tolerate any downstream sequence, since the Q/A.V/A mutant is phosphorylated as efficiently as the wild type protein. This suggests that the differences in PKA phosphorylation of different E6 PBMs are all determined by differences in the upstream sequences that make up the PKA recognition site, and are independent of any specific downstream sequences. In the case of Chk1, however, the Q/A.V/A mutant phosphorylation is

reduced compared with wild type HPV-18 E6 and, moreover, the R153A mutant abolishes Chk1-mediated phosphorylation of the PBM, suggesting that both upstream and downstream residues play critical roles in Chk1 recognition of E6. Interestingly, AKT kinase recognition does not seem to be dependent on non-canonical residues around the PBM, suggesting interesting differences in the kinase recognition of E6. Furthermore, when we analysed the phosphorylation of different high-risk HPV types with Chk1 and PKA kinase, they showed interesting differences in the levels of E6 phosphorylation. As mentioned earlier, previous studies have shown a significant role for non-canonical residues upstream of the phospho-acceptor site for PKA recognition (Boon and Banks 2013, Boon, Tomaic et al. 2015). Our results further highlight this observation, as HPV-16, -18 E6 and HPV-39 E6, which have a highly enriched stretch of arginine residues upstream, show very high levels of phosphorylation by Chk1 and PKA, whilst HPV-31, -51, -56 and -68 E6 are only weakly phosphorylated and have fewer upstream arginine residues. Interestingly, HPV-33 E6, which also has a highly arginine-enriched sequence upstream, is phosphorylated very weakly by Chk1, potentially owing to the presence of an alanine residue downstream of the phospho-acceptor site, which further supports our hypothesis. Taken together, these results highlight the importance of the PBM non-canonical residues in regulating E6 phosphorylation and how it varies for different kinases. Moreover, it also sheds insights into differential phosphorylation of different high-risk HPV types depending on the specific amino acid residues present in the motif.

2.2 Biochemical differences within the PBM allows E6 to control PDZ/14-3-3 recognition

This in depth mutational analysis of the E6 PBM also allows the efficient separation of E6's PDZ and 14-3-3 recognition. Our results demonstrate that any substitution within the PBM is deleterious to PDZ recognition, which is in support of previously reported findings. Furthermore, the R153A and ETstop mutant, which cannot be phosphorylated by Chk1 kinase, do not interact with 14-3-3, whilst the ETQstop mutant does show phosphorylation-dependent 14-3-3 association. Interestingly, the ETAA mutant

abolishes any interaction with PDZ domain-containing substrates, but retains the interaction with 14-3-3 ζ protein. Interestingly however, this mutant showed no interaction with 14-3-3 γ , suggesting various differences in the recognition of different isoforms of 14-3-3 proteins. These results are summarized in Figure 46 and this will form the basis for future studies for dissecting further the relevance of 14-3-3 interactions for different E6 activities.

One of the direct biological consequences of these activities is to regulate p53 functions. Our results demonstrate that, not only the E6 PBM, but also its phosphorylation state, plays an important role in regulating p53's transcriptional transactivation activities (see above). The HPV-56 and -68 E6 proteins, which are phosphorylated only to low levels by both Chk1 and PKA kinases, only weakly affect p53 activation of the Mdm2 promoter, whilst HPV-18 and -39 E6 proteins, which are strongly phosphorylated by both kinases, are very effective in suppressing p53 transcriptional activity under DNA damage conditions.

This indicates that the PBM interactions of E6 contribute towards the abrogation of a p53 function that is independent of the ability of E6 to degrade p53, and this functional ablation may be necessary for viral genome maintenance. E6 does this potentially through its association with 14-3-3. These studies allow us to propose a model as shown in Figure 47. However, understanding the molecular differences in association of different 14-3-3 isoforms with different HPV E6 types depending on their phospho-status will be particularly interesting. In addition, 14-3-3 proteins also have a wide range of other functions in addition to those linked with the regulation of p53 and it remains to be determined whether E6 can also modulate any of these other functions in a phosphorylation dependent manner. Finally, we should not exclude the possibility that phosphorylation of the PBM might also regulate association with other cellular proteins, and studies are currently underway to investigate this possibility further.

Taken together, these observations highlight important differences for the phosphoregulation of E6 from diverse HPV types, and suggest potentially broader effects on E6 function during the viral life cycle and during malignant progression.

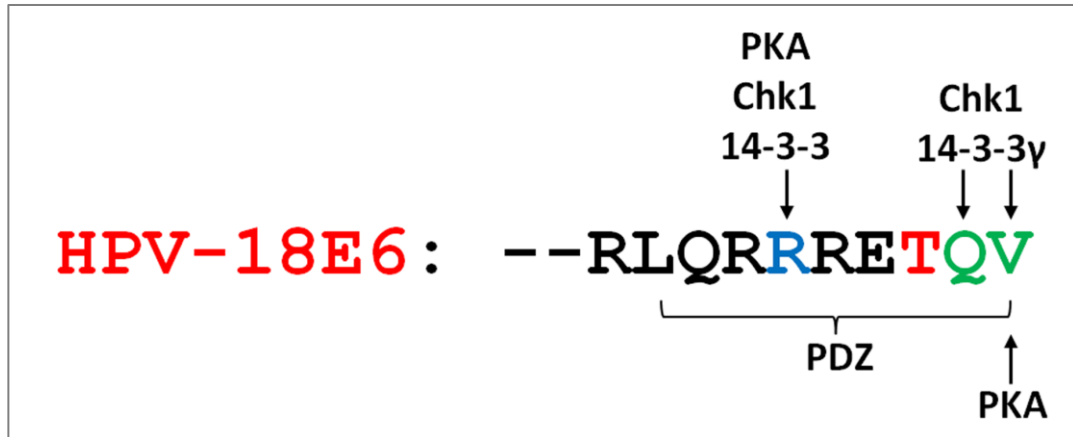


Figure 46. The Non-canonical residues around HPV-18 E6 PBM play important role in kinase recognition of E6.

A scheme showing the carboxy-terminus sequence of the HPV-18 E6 and role of specific residues in recognition by different kinases, PDZ domains and 14-3-3 recognition. The R153 residue (Blue), and the threonine phospho-acceptor site (Red) are critical for the PKA and Chk1 kinase recognition and subsequent phosphorylation-dependent 14-3-3 interaction. The residues downstream of phospho-acceptor site (Green) play important role in Chk1 kinase recognition and subsequent specific 14-3-3 interactions such as 14-3-3γ. The ultimate amino acid (Green) is important for PKA recognition but not for Chk1.

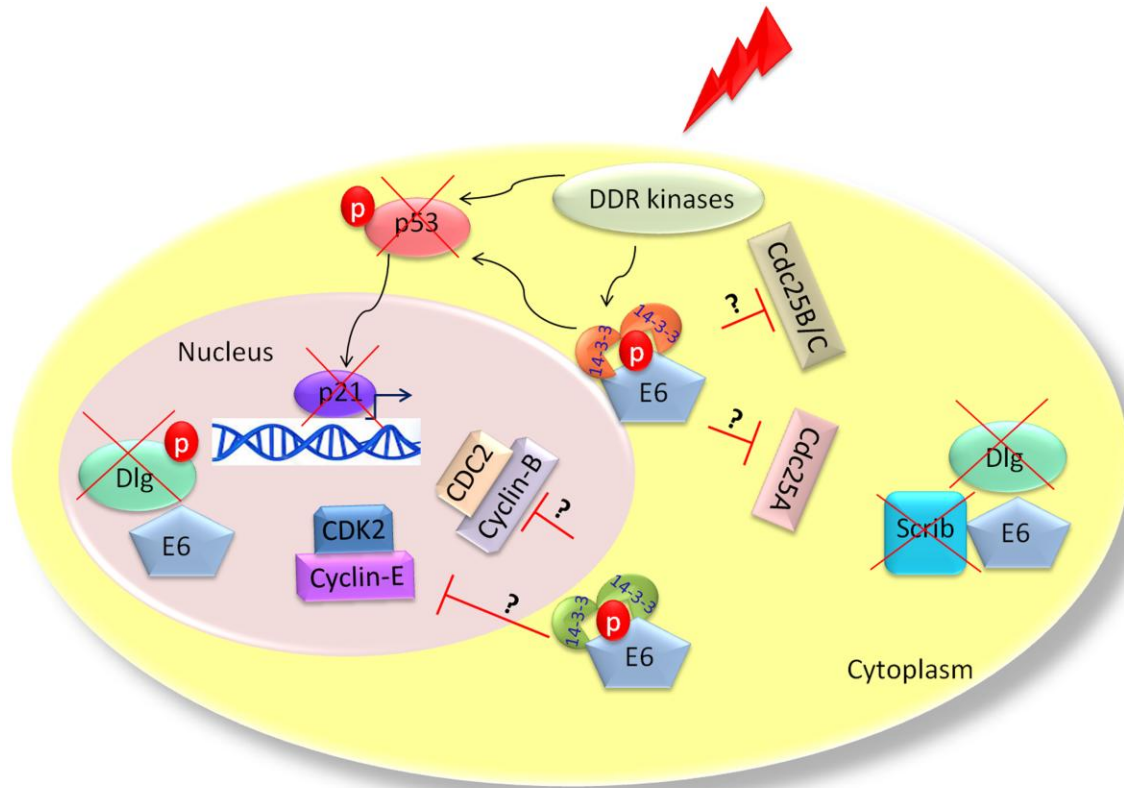


Figure 47. HPV E6 mediated perturbation of 14-3-3 and PDZ functions.

Induction of DDR signaling enhances phosphorylation of E6, which confers increased interaction with 14-3-3, resulting in 14-3-3 retention in the cytosol. Retention of 14-3-3 potentially results in retention of Cdc25A/C in the cytosol, thus inhibiting the activation of Cyclin-B and Cyclin-E respectively. 14-3-3 mediated inactivation of p53 results in inhibition of p21 transcription transactivation as well as several other p53 responsive promoters. The non-phosphorylated form of E6 interacts with hDlg (including cytoplasmic and phosphorylated nuclear forms), Scribble and other PDZ domain containing substrates, some of which are targeted for degradation.

PART:3

3. E6 over rides the biochemical regulation of E6AP

3.1 Phosphorylation of E6AP at T485 does not affect E6's ability to degrade its substrates

The cellular ubiquitin ligase, E6AP, plays a critical role in many of E6's activities. It is essential for the degradation of certain E6 substrates, it is essential for maintaining E6 protein stability and it contributes directly towards the induction of malignancy in transgenic animals (Herber, Liem et al. 1996, Shai, Nguyen et al. 2007, Shai, Pitot et al. 2010, Padash Barmchi, Gilbert et al. 2016). Furthermore, loss of its expression in cells derived from cervical cancer induces high levels of apoptosis, and therefore the E6-E6AP interaction remains an attractive target for the development of novel anti-HPV therapeutics. The recent demonstration that E6AP enzymatic activity could be modulated by PKA was therefore particularly relevant for understanding how this might affect E6 function. We show here that phosphorylation of E6AP at T485 is unlikely to directly affect the ability of E6 to target its substrates for proteasome-mediated degradation, although, depending on the specific HPV type, modulation of T485 can affect the ability of E6 to further promote E6AP auto-ubiquitination and degradation.

Previous studies had shown that the E6AP T485 residue is a phospho-acceptor site for PKA, with a non-phosphorylatable mutant, T485A, demonstrating increased levels of ubiquitination activity, both with respect to an E6AP target protein, HHR23A, and also with respect to its own auto-ubiquitination. In contrast, a phospho-mimic mutation, T485E, has apparently greatly reduced levels of enzymatic activity, again both with respect to a normal substrate and to itself. We initially confirmed these observations in two ways. First we performed *in vitro* phosphorylation assays with purified PKA and demonstrated unequivocally that the major PKA phospho-acceptor site on E6AP was at T485. Secondly, we analysed how the T485A and T485E mutants would behave with respect to a different E6AP substrate, Ring1B. In agreement with previous studies, we

found increased levels of Ring1B degradation and a modest increase in ubiquitination in the presence of the T485A mutant, but reduced levels of enzymatic activity with the T485E mutation. It should also be noted that minor differences from previously published studies (Yi, Berrios et al. 2015) could be a reflection of the fact that all our current analyses have been performed in cells in which E6AP expression was stably ablated by CRISPR/Cas9, whilst previous studies analyzed the E6AP mutants in the context of low levels of endogenously expressed wild type E6AP, thus potentially complicating the interpretation.

Importantly, when we tested the reported degradation substrates of E6AP, such as HHR23A, despite their being polyubiquitinated, we were unable to see their degradation in the presence of E6AP. Similarly, we observed no degradation of another reported substrate, PML, which suggests that care should be taken in differentiating between the *in vivo* interaction substrates and degradation substrates of E6AP.

We were then interested in determining whether the same regulation of E6AP, reported for itself and its normal substrates, also applied to substrates that were targeted as a result of the interaction with E6. We analysed two very different targets, p53 and Dlg, each of which interacts with E6 through completely different mechanisms. In both cases we found a striking similarity, in that the phospho-modulation of T485 appeared to have no effect upon the capacity of either HPV-16 or HPV-18 E6 to degrade either of these cellular proteins. Similar results were also obtained with HPV-31 E6 and p53, indicating that this also holds true for E6 proteins from multiple HPV types. This suggests that E6's recruitment of E6AP overrides the normal regulatory mechanisms that are in place to control E6AP activity.

Most interestingly, other aspects of the E6/E6AP interaction do appear to be affected by the phosphorylation status of T485. Whilst previous analyses had shown that T485A was active in auto-degradation and T485E was defective, we find that, depending upon the HPV type, these activities of E6AP are affected differently. Thus, HPV-16 E6 and HPV-31 E6 can efficiently target both the wild type E6AP and the T485E mutant for degradation, indicating that HPV-16 E6 and HPV-31 E6 can promote the auto-degradation of E6AP, regardless of the phospho-status at T485. Interestingly, HPV-16

E6 and HPV-31 E6 also seem capable of further augmenting the auto-degradation activity of the T485A mutant. In contrast, HPV-18 E6 can efficiently target WT E6AP but is defective with respect to the T485A mutant, indicating that HPV-18 E6 cannot further stimulate the already highly active auto-degradatory activity. In addition, despite T485E being very susceptible to HPV-16 E6 and HPV-31 E6 induced degradation, only a weak activity is seen with HPV-18 E6. This indicates that whilst HPV-18 E6 can still redirect an apparently inactive T458E mutant to target an E6 substrate, it cannot promote T485E auto-degradation. Taken together, these results demonstrate that different HPV E6 oncoproteins can uncouple E6AP from its normal regulation by PKA, presumably by conferring a structural modification upon E6AP itself, but that there are nonetheless quite marked differences in how different E6 oncoproteins bring this about.

3.2 E6 modulates the subcellular distribution of E6AP

Having found that E6 can significantly alter the biochemical regulation of E6AP, we also wanted to determine whether E6 could have any impact upon the subcellular distribution of phospho-E6AP. This was important since previous studies had indicated that phospho-E6AP was primarily located in the cytoplasm, whereas E6 is known to recruit E6AP to the nucleus (Daniels, Sanders et al. 1998, Vaeteewoottacharn, Chamutpong et al. 2005, Yi, Berrios et al. 2015). To do this we performed a series of immunofluorescence analyses on HeLa cells and found that in the presence of HPV-18 E6, E6AP was largely found within the nucleus, regardless of its phosphorylation status. However, when E6 was removed, total E6AP was found within the nucleus and cytoplasm, whereas the phospho-E6AP was found almost entirely within the cytoplasm. This suggests that the phosphorylation of E6AP can modulate its subcellular distribution, but this is completely overridden by E6.

Since changes in the subcellular distribution of many phosphorylated proteins are controlled by members of the 14-3-3 family (Muslin and Xing 2000), we were naturally interested in investigating whether 14-3-3 might play any role in the regulation of E6AP subcellular distribution. To do this we again made use of the 14-3-3 inhibitor, Difoepin,

which blocks 14-3-3 interaction with its target proteins (Xu, Fulop et al. 2010). Most interestingly, we found that Difoepin only had any effect on the subcellular distribution of the phospho-E6AP in the presence of E6, where, whilst having minimal effects on the total E6AP expression pattern, it induced a marked relocalisation of phospho-E6AP from the nucleus to the cytoplasm. These results indicate that E6-triggered nuclear accumulation of phospho-E6AP is partly dependent upon the activity of 14-3-3 family members. Obviously this raises a number of important questions about the precise mechanisms by which this occurs, but it is tempting to speculate that optimal recruitment of phospho-E6AP to the nucleus might require the ability of E6 to also recognize 14-3-3 proteins. Whether this requires phosphorylated E6 remains to be determined, but as the steady state levels of E6 phosphorylation are normally very low as shown above, this suggests that this activity is most likely independent of E6 phosphorylation within the PBM and indicates an indirect means of of E6AP/14-3-3 recruitment, potentially as part of a tripartite complex. Further studies will be required to clarify these precise mechanisms. It is also interesting to speculate as to why E6 might recruit phospho-E6AP to the nucleus. Under normal circumstances, this form of the protein would be inactive, and it is quite possible that in certain phases of the cell cycle, or during differentiation, E6AP might become highly phosphorylated. Therefore, without the ability of E6 to override this phospho-regulation it is quite possible that there would be times when E6 would lose much of its function. Hence the recruitment of an apparently inactive form of the protein to the nucleus and re-activating its ability to degrade E6 substrates would appear to make very good virological sense. Future studies will obviously be required to investigate these aspects further.

In conclusion, these studies demonstrate that E6 very efficiently overcomes the negative regulation of E6AP activity; however the precise mechanisms and consequences with respect to the ability of E6 to promote E6AP auto-ubiquitination vary somewhat between different HPV types, thus implying quite distinct mechanisms by which different HPV E6 oncoproteins redirect the E6AP ubiquitin ligase activity. This study also suggests that this is not a simple stimulation of an already-existing activity, but rather a redirection of E6AP activity toward itself as summarized in Figure 48.

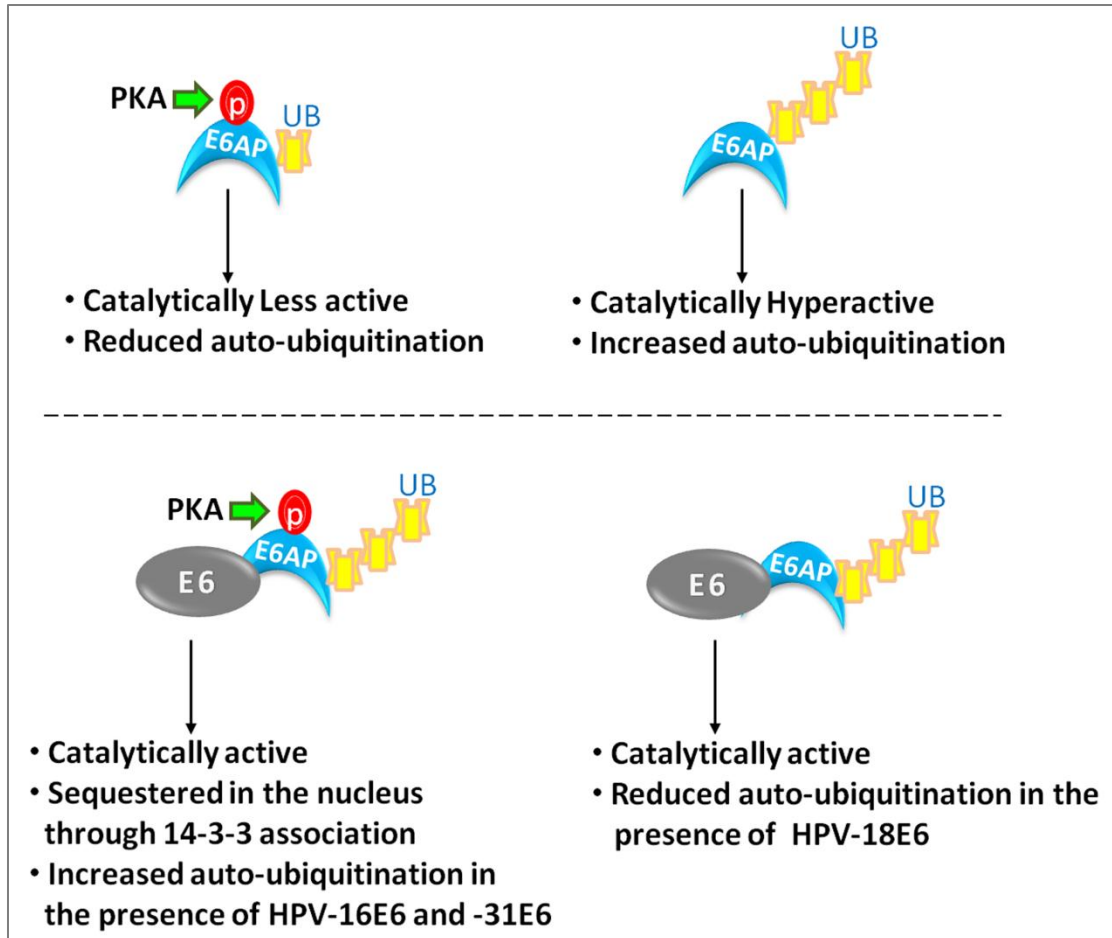


Figure 48. The HPV E6 oncoproteins override the normal phospho-regulation of E6AP.

PKA phosphorylation of E6AP at position T485 results in decreased enzymatic activity, whilst a non-phosphorylatable form of E6AP shows increased levels of ubiquitin-mediated degradation of its substrates. HPV E6 is capable of overriding this regulation and can promote degradation of its substrates regardless of the phosphorylation status of E6AP. Furthermore, E6 interaction with E6AP also significantly alters how E6AP is subject to autodegradation.

PART:4

4. HPV-31 E6 displays complex pattern of phospho-regulation

4.1 PKA phosphorylates HPV-31 E6 at a residue outside the PBM

Previously, it has been shown that deletion of the PBM in HPV-31 E6 does not affect the PKA-mediated phosphorylation of E6, suggesting that the PKA phospho-acceptor site resides outside the PBM. Additionally, the mass spectrometric analysis of PKA-phosphorylated HPV-31 E6 indicated the S82 residue as a potential phospho-acceptor site (Boon, Tomaic et al. 2015). Therefore, in this study we have further characterized the differential phosphorylation of HPV-31 E6 by PKA and AKT and attempted to determine the functional relevance of phosphorylation at S82.

Our studies demonstrate that the HPV-31 E6 displays a particularly complex pattern of phospho-regulation. We observed that the S82G mutation completely abolishes the phosphorylation by PKA, thus confirming S82 as the major phospho-acceptor site on HPV-31 E6, whilst the S82D mutation enhances the phosphorylation elsewhere in HPV-31 E6 protein. Surprisingly, when we analysed the S82D/ Δ PBM double mutant, it was still highly phosphorylated by PKA. This indicates that PKA can phosphorylate HPV-31 E6 on at least two different sites, one at S82 and another, as yet undefined, that is recognized by PKA following phosphorylation of the S82 residue, but which resides outside the PBM. The phospho-prediction analysis suggests presence of a second PKA phospho-acceptor site potentially at T58, however, further biochemical analysis needs to be done to confirm this. On the other hand, the AKT kinase phosphorylates E6 within the PBM, and deletion of the PBM completely abolishes AKT-mediated phosphorylation of E6, suggesting differential regulation of HPV-31 E6 phosphorylation with these different kinases. This is in marked contrast to HPV-16 and HPV-18 E6 where PKA and AKT both phosphorylate E6 within the PBM. In the case of Chk1 phosphorylation this is very weak with HPV-31 E6 and occurs within the PBM, but it would be interesting to investigate whether Chk2/PKA activation through oxidative stress might be able to induce phosphorylation of HPV-31 E6 at S82.

4.2 Phosphorylation at S82 most likely affects p53 degradation as a result of reduced interaction with the E6AP ubiquitin ligase

Having confirmed HPV-31 E6 S82 as a phospho acceptor site for PKA we wanted to explore how this phosphorylation event might affect E6 activity. Intriguingly, this residue seems to play an important role in the ability of HPV-31 E6 to direct the degradation of p53. Both the A and G substitution mutants induce the degradation of p53 with an efficiency similar to that of wild type E6, but the phospho-mimic substitution of S82D greatly reduces the ability of HPV-31 E6 to degrade p53. I also wanted to examine whether this residue was critical in E6 from other HPV types and so also analysed the equivalent S82D mutation in HPV-18 E6. In this case, the HPV-18 E6 S82D substitution mutant was able to induce p53 degradation as efficiently as the wild type HPV-18 E6. These results demonstrate that inserting a phospho-mimic mutation at this site is not producing a general disruption in the overall basic structure of E6, but rather this seems specific for HPV-31 E6. Whether other HPV E6 proteins can also be phosphorylated at this residue with similar consequences remains to be determined.

I then proceeded to investigate the molecular basis for loss of p53 degradation with the S82D mutant. Clearly one contributing explanation is the fact that this mutant appears to be much more weakly expressed than the wild type HPV-31 E6 protein in western blot analysis, and this alone might seem sufficient to explain loss of p53 degradation activity. However, when these mutants were analysed by immunofluorescence, it can clearly be seen that the S82D can co-localize with p53, whereas neither the wild type E6 or S82A mutant could be found co-localising with p53 in these assays. This is consistent with the phospho-dead mutants of E6 being as efficient as wild type E6 in inducing p53 degradation, but that the phospho-mimic mutant, as well as being unstable, is also inherently defective in its ability to degrade p53. This might suggest that phosphorylation at S82 does not simply result in E6 instability, but also in a reduced ability to target p53 for degradation. To further investigate the mechanisms behind this, we analysed the ability of these mutants to interact with p53 and E6AP. We observed that there are minimal differences between wild type and mutant E6s in their ability to associate with p53. However, there is a striking decrease in the ability of

the S82D mutant to interact with E6AP, compared with the S82A or S82G mutant or the wild type E6. These results suggest that addition of a negative charge, most likely through phosphorylation by PKA on the HPV-31 E6 S82 residue, controls the interaction with E6AP, thereby modulating the levels of E6 expression and simultaneously reducing the ability to degrade p53 (Summarized in Figure 49). This has important implications for how we view the regulation of E6 protein from diverse HPV types, and suggests that phosphorylation events, depending on the specific HPV E6 protein, can have broader effects on E6 structure and function other than simple regulation of the E6 PBM.

These results raise a number of interesting questions, the most important being when does HPV-31 E6 become phosphorylated. Based on our studies with HPV-18 E6 in HeLa cells it seems unlikely that HPV-31 E6 would be phosphorylated during a normal cell cycle, since if this was the case, we would have expected to see PKA phosphorylating HPV-18 E6 in the PBM during our cell cycle analyses, since we know that HPV-18 E6 is an excellent substrate for PKA. This raises two other possibilities. The first is that that phosphorylation takes place also upon induction of a DDR through Chk2 activation of PKA, in manner similar to that seen for phosphorylation of the HPV-18 E6 PBM. However, this does seem rather counter-intuitive, as it would imply less E6 and less effect upon p53 just at a point when a DDR was being activated. One possibility might be that the phosphorylation at S82 under these circumstances confers interaction with certain 14-3-3 isoforms, which might in turn compensate for loss of interaction with E6AP, and might allow inhibition of p53 transcriptional activity in a manner similar to that seen with PBM phosphorylated HPV-18 E6. The second possibility is that the S82 residue becomes phosphorylated during differentiation at a point when high level expression of E6 is no longer required. There are reports of high levels of PKA activity in the upper layers of terminally differentiating keratinocytes (Delury, Marsh et al. 2013) so this would be consistent with this hypothesis. To resolve these questions, it will be necessary to look at the role of the S82 mutants in the context of the whole HPV-31 genome in combination with studies to monitor phosphorylation at this specific residue during different stages of the viral life cycle and post induction of a DDR.

Summary

The results presented in this thesis reveal a very complex pattern of HPV E6 phosphorylation events in response to various forms of different stresses, the precise form of which varies between different HPV E6 proteins, and results in similarly different functional readouts. This can be combined also with the effects of phosphorylation of a key cellular interaction partner, E6AP, which can also potentially impact upon E6 function in vivo. These phosphorylation events begin to form a picture of a highly dynamic set of E6 interactions, involving various PDZ domain containing substrates, switching between different 14-3-3 isoforms, which in turn can modulate E6AP association, substrate degradation and E6 stability. As can be seen in Figure 50 these different activities and pathways are potentially highly amenable to inhibition via various small molecules, the consequences of which might have profound effects upon continuing cancer growth and the HPV life cycle.

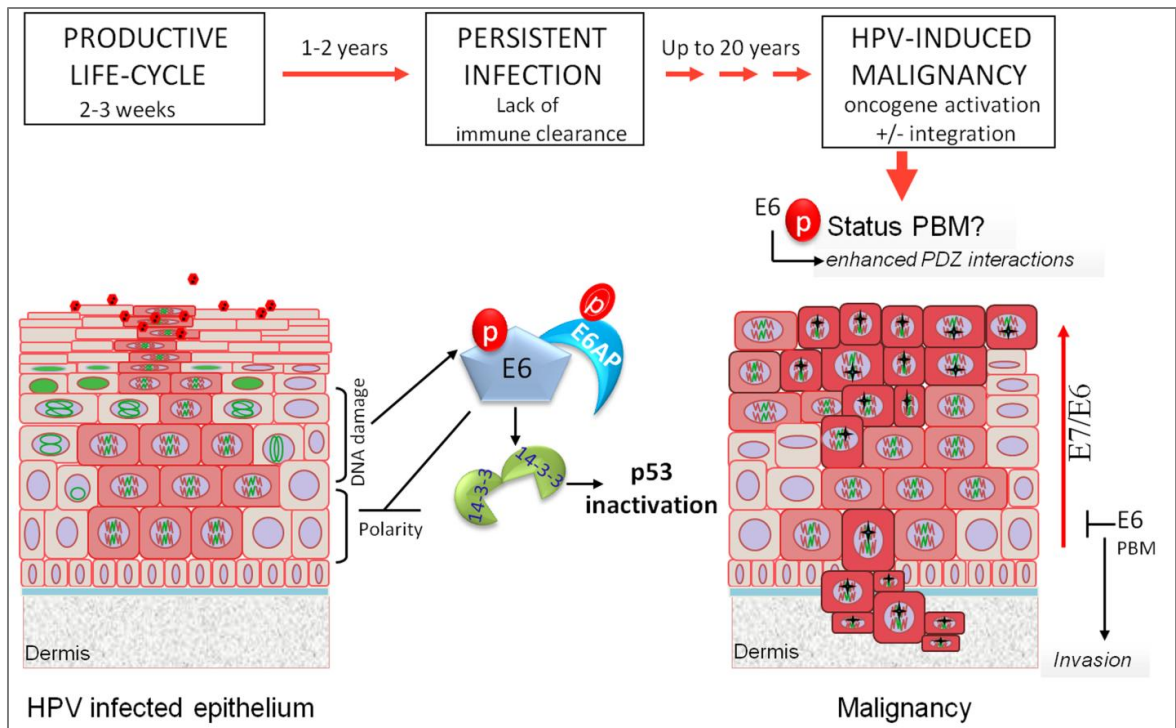


Figure 50. Summary of the roles of HPV E6 phosphorylation and some of the associated activities during the viral life cycle and progression to malignancy.

The HPV E6 through the PBM perturbs cell polarity by degrading PDZ-containing proteins and thus, allowing cells to differentiate to suprabasal layers. The rapid replication of the viral genome during the amplification phase creates aberrant DNA structures that activate DNA damage response signaling. Activation of ATR/Chk1 and ATM/Chk2 kinases further induce E6 phosphorylation within the PBM. This allows E6 to interact with 14-3-3 family proteins, thus perturbing p53 transcriptional transactivation. E6 recruits an inactive form of pE6AP to the nucleus and re-activates its ability to degrade E6 substrates. The potential roles of phosphorylation of E6 during development of malignancy still remains to be determined.

List of Publications

1. **Thatte, J.** and L. Banks (2017). "Human Papillomavirus 16 (HPV-16), HPV-18, and HPV-31 E6 Override the Normal Phosphoregulation of E6AP Enzymatic Activity." *J Virol* 91(22).
2. **Thatte, J.**, P. Massimi, M. Thomas, S. S. Boon and L. Banks (2018). "The Human Papillomavirus E6 PDZ Binding Motif Links DNA Damage Response Signaling to E6 Inhibition of p53 Transcriptional Activity." *J Virol* 92(16).
3. **Thatte J.** Vats A. and Banks L., 'The HPV E6 association with the PDZ proteins and 14-3-3 family proteins is differentially regulated in different HPV types.' (Manuscript ready to submit).
4. **Thatte J. Massimi P.** and Banks L. 'The HPV-31 E6 displays a complex pattern of phospho-regulation'. (Manuscript submitted to *Virology*).
5. Ganti K, Broniarczyk J, Manoubi W, Massimi P, Mittal S, Pim D, Szalmas A, **Thatte J**, Thomas M, Tomaić V, Banks L., The Human Papillomavirus E6 PDZ Binding Motif: From Life Cycle to Malignancy. *Viruses*. 2015.

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